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(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : KHAN, Mohammed, Anjam [GB/GB]; HORMAECHE, Carlos, Estenio [GB/GB]; VILLARREAL-RAMOS, Bernardo [MX/GB]; Cambridge University Department of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB). CHATFIELD, Steven, Neville [GB/GB]; Medeva Vaccine Research Unit, Department of Biochemistry, Imperial College of Science & Technology, London SW7 2AY (GB). DOUGAN, Gordon [GB/GB]; Department of Biochemistry, Imperial College of Science & Technology, London SW7 2AY (GB).

(54) Title: EXPRESSION OF RECOMBINANT FUSION PROTEINS IN ATTENUATED BACTERIA

(57) Abstract

The invention provides a DNA molecule comprising a promoter sequence operably linked to a DNA sequence encoding first and second proteins linked by a hinge region wherein in that the promoter sequence can be one having activity which is induced in response to a change in the surrounding environment and the first protein can be Tetanus toxin C fragment or one or more epitopes thereof. The invention also provides intermediate molecules having a promoter operably linked to a DNA sequence encoding a first antigenic sequence and a hinge region, and at or adjacent the 3'-end thereof one or more restriction sites for the introduction of a second antigenic sequence. In addition, the invention provides replicable expression vectors containing the DNA fusion proteins expressed therefrom, bacterial transformed with the vectors and the use of the bacteria, in vaccines.

| Pst | BamHI | EcoRV | HindIII | SpeI | Stop | BamHI |
| --- | --- | --- | --- | --- | --- | --- |

---HINGE----    TCTAGA GGATCC GATATC AAGCTT ACTAGT TAA TGATC
                   AGATCT CCTAGG CTATAG TTCGAA TGATCA ATT ACTAG
                              (SEQ ID NO: 19)

---GPGP ----       S   R   G   S   D   I   K   L   T   S *

                      (SEQ ID NO: 20)

# EXPRESSION OF RECOMBINANT FUSION PROTEINS IN ATTENUATED BACTERIA

This invention relates to DNA constructs, replicable expression vectors containing the constructs, attenuated bacteria containing the constructs and vaccines containing the said bacteria.

In recent years, there has emerged a new generation of live oral salmonella vaccines based upon strains of Salmonella which have been attenuated by the introduction of a non-reverting mutation in a gene in the aromatic biosynthetic pathway of the bacterium. Such strains are disclosed, for example, in EP-A-0322237. The aforesaid live oral salmonella vaccines are showing promise as vaccines for salmonellosis in man and animals, and they can also be used effectively as carriers for the delivery of heterologous antigens to the immune system. Combined salmonella vaccines have been used to deliver antigens from viruses, bacteria, and parasites, eliciting secretory, humoral and cell-mediated immune responses to the recombinant antigens. Combined salmonella vaccines show great potential as single dose oral multivaccine delivery systems [C. Hormaeche et al, FEMS Symposium No. 63, Plenum,

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New York; pp 71-83, 1992].

There are problems to be overcome in the development
of combined salmonella vaccines.  A major consideration is
obtaining a high level of expression of the recombinant
antigen in the salmonella vaccine so that it will be
sufficient to trigger an immune response.   However,
unregulated high level expression of foreign antigens can
be toxic and affect cell viability [I. Charles and G.
Dougan, TIBTECH 8, pp 117-21, 1990], rendering the vaccine
ineffective or causing loss of the recombinant DNA.
Several possible solutions to this problem have been
described, such as expression from plasmids carrying
essential genes, "on-off" promoters or incorporation of the
foreign genes into the salmonella chromosome.

An alternative approach to overcoming the aforesaid
problem would be to use a promoter which is inducible in
vivo, and one such promoter is the E.coli nitrite reductase
promoter nirB which is induced under anaerobiosis and has
been used in biotechnology for the production of tetanus
toxin fragment C (TetC) of Clostridium tetani [M.D. Oxer et
al Nucl. Ac. Res., 19, pp 2889-92, 1991].   It has
previously been found by the inventors of this application
(S.N. Chatfield et al Bio/Technology, Vol. 10, pp 888-92
1992) that an Aro Salmonella harbouring a construct
expressing TetC from the nirB promoter (pTETnir15) elicited
very high anti-tetanus antibody responses in mice.   The
article by Chatfield et al was published after the priority
date of this application.

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However, we have also found that when it was attempted to express the P28 antigen from _Schistosoma mansoni_ alone from _nirB_, the resulting construct was not immunogenic.

Tetanus toxoid has been extensively used as an adjuvant for chemically coupled guest epitopes [D.A. Herrington _et al._ Nature, 328, pp 257-9 1987]. The potent immunogenicity of TetC in _Salmonella_ suggested to us that it may be possible to exploit this character to promote the immune response of the guest peptides or proteins. However, fusing two proteins together often leads to an incorrectly folded chimaeric protein which no longer retains the properties of the individual components. For example the B subunit of the _Vibrio cholerae_ (CT-B) and _E.coli_ (LT-B) enterotoxins are powerful mucosal immunogens but genetic fusions to these subunits can alter the structure and properties of the carrier and hence their immunogenicity [see M. Sandkvist _et al._ J. Bacteriol 169, pp 4570-6, 1987, Clements 1990 and M. Lipscombe _et al_ [Mol. Microbiol 5, pp 1385 1990]. Moreover, many heterologous genes expressed in bacteria are not produced in soluble properly folded, or active forms and tend to accumulate as insoluble aggregates [see C. Schein _et al._ Bio/Technology 6, pp 291-4, 1988 and R. Halenbeck _et al_; Bio/Technology 7, pp 710-5, 1989].

It is an object of the invention to overcome the aforementioned problems.

We have now found that efficient expression of recombinant antigens, and in particular fusion proteins,

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can be achieved in bacteria such as <u>salmonellae</u>, by the use of an inducible promoter such as nirB and by incorporating a flexible hinge region between two antigenic components of the fusion protein. The resulting recombinant antigens have been shown to have good immunogenicity. It has also been found, surprisingly, that enhanced expression of a protein can be obtained when a gene coding for the protein is linked to the gene for tetanus toxin C fragment.

Accordingly, in a first aspect, the present invention provides a DNA construct comprising a promoter sequence operably linked to a DNA sequence encoding first and second proteins linked by a hinge region, characterised in that the promoter sequence is one having activity which is induced in response to a change in the surrounding environment.

In another aspect, the invention provides a DNA construct comprising a promoter sequence operably linked to a DNA sequence encoding linked first and second proteins, wherein the first heterologous protein is an antigenic sequence comprising tetanus toxin fragment C or one or more epitopes thereof.

In a further aspect, the invention provides a replicable expression vector, suitable for use in bacteria, containing a DNA construct as hereinbefore defined.

In a another aspect, the invention provides a fusion protein, preferably in substantially pure form, the fusion protein comprising linked (e.g. by a hinge region) first and second proteins, the fusion protein being expressed by

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a replicable expression vector as hereinbefore defined.

In a further aspect the invention provides a process for the preparation of an attenuated bacterium which comprises transforming an attenuated bacterium with a DNA construct as hereinbefore defined.

The invention also provides a vaccine composition comprising an attenuated bacterium, or a fusion protein, as hereinbefore defined, and a pharmaceutically acceptable carrier.

The first and second proteins are preferably heterologous proteins and in particular can be polypeptide immunogens; for example they may be antigenic sequences derived from a virus, bacterium, fungus, yeast or parasite. In particular, it is preferred that the first said protein is an antigenic sequence comprising tetanus toxin fragment C or epitopes thereof.

The second protein is preferably an antigenic determinant of a pathogenic organism. For example, the antigenic determinant may be an antigenic sequence derived from a virus, bacterium, fungus, yeast or parasite.

Examples of viral antigenic sequences for the first and/or second heterologous proteins are sequences derived from a type of human immuno-deficiency virus (HIV) such as HIV-1 or HIV-2, the CD4 receptor binding site from HIV, for example from HIV-1 or -2., hepatitis A or B virus, human rhinovirus such as type 2 or type 14, Herpes simplex virus, poliovirus type 2 or 3, foot-and-mouth disease virus (FMDV), rabies virus, rotavirus, influenza

virus, coxsackie virus, human papilloma virus (HPV), for
example the type 16 papilloma virus, the E7 protein
thereof, and fragments containing the E7 protein or its
epitopes; and simian immunodeficiency virus (SIV).
Examples of antigens derived from bacteria are those
derived from <u>Bordetella pertussis</u> (e.g. P69 protein and
filamentous haemagglutinin (FHA) antigens), <u>Vibrio
cholerae</u>, <u>Bacillus anthracis</u>, and <u>E.coli</u> antigens such as
<u>E.coli</u> heat Labile toxin B subunit (LT-B), <u>E.coli</u> K88
antigens, and enterotoxigenic <u>E.coli</u> antigens.    Other
examples of antigens include the cell surface antigen CD4,
<u>Schistosoma mansoni</u> P28 glutathione S-transferase antigens
(P28 antigens) and antigens of flukes, mycoplasma,
roundworms, tapeworms, <u>Chlamydia trachomatis</u>, and malaria
parasites, eg. parasites of the genus plasmodium or
babesia, for example <u>Plasmodium falciparum,</u> and peptides
encoding immunogenic epitopes from the aforementioned
antigens.

    Particular antigens include the full length
<u>Schistosoma mansoni</u> P28, and oligomers (e.g. 2, 4 and 8-
mers) of the immunogenic P28 aa 115-131 peptide (which
contains both a B and T cell epitope), and human papilloma
virus E7 protein, Herpes simplex antigens, foot and mouth
disease virus antigens and simian immunodeficiency virus
antigens.

    The promoter sequence is one having activity which is
induced in response to a change in the surrounding
environment, and an example of such a promoter sequence is

one which has activity which is induced by anaerobic
conditions. A particular example of such a promoter
sequence is the nirB promoter which has been described,
for example in International Patent Application
PCT/GB92/00387. The nirB promoter has been isolated from
E.coli, where it directs expression of an operon which
includes the nitrite reductase gene nirB (Jayaraman et al,
J. Mol. Biol. 196, 781-788, 1987), and nirD, nirC, cysG
(Peakman et al, Eur. J. Biochem. 191, 315-323, 1990). It
is regulated both by nitrite and by changes in the oxygen
tension of the environment, becoming active when deprived
of oxygen, (Cole, Biochem, Biophys. Acta. 162, 356-368,
1968). Response to anaerobiosis is mediated through the
protein FNR, acting as a transcriptional activator, in a
mechanism common to many anaerobic respiratory genes.

By deletion and mutational analysis the part of the
promoter which responds solely to anaerobiosis has been
isolated and by comparison with other
anaerobically-regulated promoters a consensus FNR-binding
site has been identified (Bell et al, Nucl, Acids. Res. 17,
3865-3874, 1989; Jayaraman et al, Nucl, Acids, Res. 17,
135-145, 1989). It has also been shown that the distance
between the putative FNR-binding site and the -10 homology
region is critical (Bell et al, Molec. Microbiol.4,
1753-1763, 1990). It is therefore preferred to use only
that part of the nirB promoter which responds solely to
anaerobiosis. As used herein, references to the nirB
promoter refer to the promoter itself or a part or

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derivative thereof which is capable of promoting expression
of a coding sequence under anaerobic conditions. The
preferred sequence, and which contains the nirB promoter
is: AATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATCGTTAAGG
TAGGCGGTAGGGCC (SEQ ID NO: 1)

The hinge region is a region designed to promote the
independent folding of both the first and second proteins
by providing both spatial and temporal separation between
the domains.

The hinge region typically is a sequence encoding a
high proportion of proline and/or glycine amino acids. The
hinge region may be composed entirely of proline and/or
glycine amino acids. The hinge region may comprise one or
more glycine-proline dipeptide units.

The hinge region may, for example, contain up to about
fifteen amino acids, for example at least 4 and preferably
6-14 amino acids, the number of amino acids being such as
to impart flexibility between the first and second
proteins.

In one embodiment, the hinge region can correspond
substantially to the hinge domain of an antibody
immunoglobulin. The hinge regions of IgG antibodies in
particular are rich in prolines [T.E. Michaelson et al. J.
Biol. Chem. 252, 883-9 1977], which are thought to provide
a flexible joint between the antigen binding and tail
domains.

Without wishing to be bound by any theory, the
prolines are thought to form the rigid part of the hinge as

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the ring structure characteristic of this amino acid hinders rotation around the peptide bond that connects the proline residue with an adjacent amino acid. This property is thought to prevent proline, and adjacent residues, from adopting the ordered structure of an alpha helix or beta strand. Flexibility is thought to be imparted by glycine, the simplest amino acid, with very limited steric demands. Glycine is thought to function as a flexible elbow in the hinge. Other amino acids may be substituted for glycine, particularly those without bulky side-chains, such as alanine, serine, asparagine and threonine.

In one preferred embodiment, the hinge region is a chain of four or more amino acids defining the sequence

$$-[X]_p-Pro-[Y]_q-Pro-[Z]_r-$$
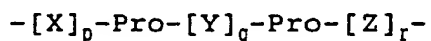
wherein Pro is proline, X and Y are each glycine, or an amino acid having a non-bulky side chain; Z is any amino acid; p is a positive integer; q is a positive integer of from one to ten; and r is zero or a positive integer greater than zero.

The hinge region can be a discrete region heterologous to both the first and second proteins or can be defined by a carboxy-end portion of the first protein or an amino-end portion of the second protein.

Codons which are infrequently utilised in E.coli [H. Grosjean et al, Gene 18, 199-209, 1982] and Salmonella are selected to encode for the hinge, as such rare codons are thought to cause ribosomal pausing during translation of the messanger RNA and allow for the correct folding of

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polypeptide domains [I.J. Purvis *et al.* J. Mol. Biol. <u>193</u>, 413-7 1987]. In addition, where possible restriction enzymes are chosen for the cloning region which, when translated in the resulting fusion, do not encode for bulky or charged side-groups.

In a most preferred aspect, the present invention provides a DNA molecule comprising the <u>nirB</u> promoter operably linked to a DNA sequence encoding first and second polypeptide immunogens linked by a hinge region, wherein the first polypeptide immunogen comprises tetanus toxin fragment C or epitopes thereof.

In another preferred aspect of the invention, there is provided a replicable expression vector, suitable for use in bacteria, containing the <u>nirB</u> promoter sequence operably linked to a DNA sequence encoding first and second polypeptide immunogens linked by a hinge region, wherein the first polypeptide immunogen comprises tetanus toxin fragment C or epitopes thereof.

It has been found that by providing a DNA sequence encoding tetanus toxin fragment C (TetC) linked via a hinge region to a second sequence encoding an antigen, the expression of the sequence in bacterial cells is enhanced relative to constructs wherein the fragment C and hinge region are absent. For example, the expression level of the full length P28 protein of <u>S. mansoni</u> when expressed as a fusion to TetC was greater than when the P28 protein was expressed alone from the <u>nirB</u> promoter. The TetC fusions to the full length P28 protein of <u>S. mansoni</u> and its tandem

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epitopes were all soluble and expressed in both E.coli and S.typhimurium. In addition, the TetC-P28 fusion protein was capable of being affinity purified by a glutathione agarose matrix, suggesting that the P28 had folded correctly to adopt a conformation still capable of binding to its natural substrate.

Stable expression of the first and second heterologous proteins linked by the hinge region can be obtained in vivo. The heterologous proteins can be expressed in an attenuated bacterium which can thus be used as a vaccine.

The attenuated bacterium may be selected from the genera Salmonella, Bordetella, Vibrio, Haemophilus, Neisseria and Yersinia. Alternatively, the attenuated bacterium may be an attenuated strain of enterotoxigenic Escherichia coli. In particular the following species can be mentioned: S.typhi - the cause of human typhoid; S.typhimurium - the cause of salmonellosis in several animal species; S.enteritidis - a cause of food poisoning in humans; S.choleraesuis - a cause of salmonellosis in pigs; Bordetella pertussis - the cause of whooping cough; Haemophilus influenzae - a cause of meningitis; Neisseria gonorrhoeae - the cause of gonorrhoea; and Yersinia - a cause of food poisoning.

Attenuation of the bacterium may be attributable to a non-reverting mutation in a gene in the aromatic amino acid biosynthetic pathway of the bacterium. There are at least ten genes involved in the synthesis of chorismate, the branch point compound in the aromatic amino acid

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biosynthetic pathway. Several of these map at widely differing locations on the bacterial genome, for example aroA (5-enolpyruvylshikimate-3-phosphate synthase), aroC (chorismate synthase), aroD (3-dihydroquinate dehydratase) and aroE (shikimate dehydrogenase). A mutation may therefore occur in the aroA, aroC, aroD, or aroE gene.

Preferably, however, an attenuated bacterium harbours a non-reverting mutation in each of two discrete genes in its aromatic amino acid biosynthetic pathway. Such bacteria are disclosed in EP-A-0322237. Double aro mutants which are suitable are aroA aroC, aroA aroD, and aroA aroE. Other bacteria having mutations in other combinations of the aroA, aroC, aroD and aroE genes are however useful. Particularly preferred are Salmonella double aro mutants, for example double aro mutants of S.typhi or S.typhimurium, in particular aroA aroC, aroA aroD and aroA aroE mutants. Alternatively, the attenuated bacterium may harbour a non-reverting mutation in a gene concerned with the regulation of one or more other genes (EP-A-0400958). Preferably the mutation occurs in the ompR gene or another gene involved in regulation. There are a large number of other genes which are concerned with regulation and are known to respond to environmental stimuli (Ronson et al, Cell 49, 579-581).

This type of attenuated bacterium may harbour a second mutation in a second gene. Preferably the second gene is a gene encoding for an enzyme involved in an essential biosynthetic pathway, in particular genes

involved in the pre-chrorismate pathway involved in the biosynthesis of aromatic compounds. The second mutation is therefore preferably in the aroA, aroC or aroD gene.

Another type of attenuated bacterium is one in which attenuation is brought about by the presence of a non-reverting mutation in DNA of the bacterium which encodes, or which regulates the expression of DNA encoding, a protein that is produced in response to environmental stress. Such bacteria are disclosed in WO 91/15572. The non-reverting mutation may be a deletion, insertion, inversion or substitution. A deletion mutation may be generated using a transposon.

An attenuated bacterium containing a DNA construct according to the invention can be used as a vaccine. Fusion proteins (preferably in substantially pure form) expressed by the bacteria can also be used in the preparation of vaccines. For example, a purified TetC-P28 fusion protein has been found to be immunogenic on its own. In a further aspect therefore, the invention provides a vaccine composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, an attenuated bacterium or fusion protein as hereinbefore defined.

The vaccine may comprise one or more suitable adjuvants.

The vaccine is advantageously presented in a lyophilised form, for example in a capsular form, for oral administration to a patient. Such capsules may be provided

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with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", Cellulose acetate, Cellulose acetate phthalate or Hydroxypropylmethyl Cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in buffer at a suitable pH to ensure the viability of the organisms. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared for parenteral administration, intranasal administration or intramammary administration.

The attenuated bacterium containing the DNA construct of the invention may be used in the prophylactic treatment of a host, particularly a human host but also possibly an animal host. An infection caused by a micro-organism, especially a pathogen, may therefore be prevented by administering an effective dose of an attenuated bacterium according to the invention. The bacterium then expresses a heterologous protein or proteins capable of raising antibody to the micro-organism. The dosage employed will be dependent on various factors including the size and weight of the host, the type of vaccine formulated and the nature of the heterologous protein.

An attenuated bacterium according to the present invention may be prepared by transforming an attenuated

bacterium with a DNA construct as hereinbefore defined. Any suitable transformation technique may be employed, such as electroporation. In this way, an attenuated bacterium capable of expressing a protein or proteins heterologous to the bacterium may be obtained. A culture of the attenuated bacterium may be grown under aerobic conditions. A sufficient amount of the bacterium is thus prepared for formulation as a vaccine, with minimal expression of the heterologous protein occurring.

The DNA construct may be a replicable expression vector comprising the nirB promoter operably linked to a DNA sequence encoding the tetanus toxin C fragment or epitopes thereof and the second heterologous protein, linked by a hinge region. The nirB promoter may be inserted in an expression vector, which already incorporates a gene encoding one of the heterologous proteins (e.g. tetanus toxin C fragment), in place of the existing promoter controlling expression of the protein. The hinge region and gene encoding the second heterologous protein (e.g. an antigenic sequence) may then be inserted. The expression vector should, of course, be compatible with the attenuated bacterium into which the vector is to be inserted.

The expression vector is provided with appropriate transcriptional and translational control elements including, besides the nirB promoter, a transcriptional termination site and translational start and stop codons. An appropriate ribosome binding site is provided. The

vector typically comprises an origin of replication and, if desired, a selectable marker gene such as an antibiotic resistance gene. The vector may be a plasmid.

The invention will now be illustrated but not limited, by reference to the following examples and the accompanying drawings, in which:

Figure 1 is a schematic illustration of the construction of an intermediate plasmid pTECH1 in accordance with one aspect of the invention.

Figure 2 is a schematic illustration of the construction of a second intermediate plasmid pTECH2.

Figure 3 is a schematic illustration of the construction of a plasmid of the invention using the intermediate plasmid of Figure 2 as the starting material. In Figure 3 B= BamHI, E = EcoRV; H = HindIII; X = XbaI; S = SpeI.

Figure 4 is a schematic illustration of the construction of a plasmid containing repeating epitopes (repitopes).

Figure 5 illustrates antibody responses against recombinant S. mansoni protein P28 as detected by ELISA in mice inoculated intravenously with SL3261, SL3261(pTETnir15), SL3261 (pTECH2), SL3261(pTECH2-monomer), SL3261 (pTECH2-dimer), SL3261(pTECH2-tetramer), SL3261(pTECH2-octamer), and SL3261(pTECH1-P28). In Figure 5 the results are expressed as OD in individual mice at intervals after immunisation.

Figure 6 illustrates antibody responses against TetC

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as detected by ELISA in mice inoculated as in Figure 5.

Figure 7 illustrates antibody responses against peptide 115-131 of the P28 protein coupled to ovalbumin as detected by ELISA in mice inoculated intravenously with SL3261, SL3261(pTECH2), SL3261(pTECH2-monomer), SL3261(pTECH2-dimer), SL3261(pTECH2-tetramer), and

SL3261(pTECH2-octamer).

Figure 8 illustrates antibody responses against TetC as detected by ELISA from mice inoculated orally with SL3261(pTECH1-P28).

Figure 9 illustrates antibody responses against recombinant P28 as detected by ELISA in mice inoculated as in Figure 8.

Figure 10 illustrates schematically the preparation of various constructs from the pTECH2 intermediate plasmid.

Figure 11 illustrates schematically the structure of tripartite protein structures ("heteromers") prepared using pTECH2.

Figure 12 shows the DNA sequence of the vector pTECH1. (SEQ ID NO: 17).

Figure 13 shows the DNA sequence of the vector pTECH2. (SEQ ID NO: 18).

Figure 14 illustrates, schemetically, the restriction sites on the vector pTECH2.

EXAMPLE 1

Preparation of pTECH1

The preparation of pTECH1, a plasmid incorporating the

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<u>nirB</u> promoter and TetC gene, and a DNA sequence encoding a hinge region and containing restriction endonuclease sites to allow insertion of a gene coding for a second or guest protein, is illustrated in Figure 1.   Expression plasmid pTETnir15, the starting material shown in Figure 1, was constructed from pTETtac115 (Makoff *et al*, Nucl. Acids Res. <u>17</u> 10191-10202, 1989); by replacing the <u>EcoRI-ApaI</u> region (1354bp) containing the <u>lacI</u> gene and <u>tac</u> promoter with the following pair of oligos 1 and 2:

Oligo-1 5'AATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAAT

Oligo-2      3'-GTCCATTTAAACTACATGTAGTTTACCATGGGGAACGACTTA

CGTTAAGGTAGGCGGTAGGGCC-3'       (SEQ ID NO: 2)

GCAATTCCATCCGCCATC-5'       (SEQ ID NO: 3)

The oligonucleotides were synthesised on a Pharmacia Gene Assembler and the resulting plasmids confirmed by sequencing (Makoff *et al*, Bio/Technology <u>7</u>, 1043-1046, 1989).

The pTETnir15 plasmid was then used for construction of the novel pTECH1 plasmid incorporating a polylinker region suitable as a site for insertion of heterologous DNA to direct the expression of fragment C fusion proteins. pTETnir15 is a known pAT153-based plasmid which directs the expression of fragment C.  However, there are no naturally occurring convenient restriction sites present at the 3'-end of the TetC gene.  Therefore, target sites, preceded by a hinge region, were introduced at the 3'-end of the TetC coding region by means of primers tailored with "add-on" adapter sequences (Table 1), using the polymerase

chain reaction (PCR) [K. Mullis *et al*, Cold Spring Harbor
Sym. Quant. Biol. <u>51</u>, 263-273 1986]. Accordingly, pTETnir15
was used as a template in a PCR reaction using primers
corresponding to regions covering the SacII and BamHI
sites. The anti-sense primer in this amplification was
tailored with a 38 base 5'-adaptor sequence. The
anti-sense primer was designed so that a sequence encoding
novel <u>XbaI</u>, <u>Spe</u>I and <u>Bam</u>HI sites were incorporated into the
PCR product. In addition, DNA sequences encoding
additional extra amino acids including proline were
incorporated (the hinge regions) and a translation stop
codon signal in frame with the fragment C open reading
frame.

The PCR product was gel-purified and digested with
<u>Sac</u>II and <u>Bam</u>HI, and cloned into the residual 2.8 kb vector
pTETnir15 which had previously been digested by <u>Sac</u>II and
<u>Bam</u>HI. The resulting plasmid purified from transformed
colonies and named pTECH 1 is shown in Figure 1.
Heterologous sequences such as the sequence encoding the
<u>Schistosoma</u> <u>mansoni</u> P28 glutathione S-transferase (P28)
were cloned into the <u>Xba</u>I <u>Spe</u>I and <u>Bam</u>HI sites in
accordance with known methods.

EXAMPLE 2

Construction of pTECH2

To further improve the utility of pTECH1, a short
linker sequence was introduced between the <u>Xba</u>I and <u>Bam</u>HI
sites in pTECH1 to allow the directional cloning of
oligonucleotides and to also facilitate the construction of

multiple tandem epitopes, ("repitopes") (Figure 2). Two
complementary oligonucleotides were synthesised bearing the
restriction enzyme target sites for BamHI, EcoRV, HindIII,
SpeI, followed by a translational stop codon (Table 1).
The oligonucleotides were tailored with XbaI and BamHI
cohesive ends; however, the BamHI target sequence was
designed to include a mismatch and, upon cloning, this
restriction site in pTECH1 is destroyed. This version of
the vector was designated pTECH2.

EXAMPLE 3

Construction of pTECH1-P28

A P28 gene expression cassette was produced by PCR
using pUC19-P28 DNA (a kind gift from Dr R Pierce, Pasteur
Institute, Lille) as template. Oligonucleotide primers
were designed to amplify the full length P28 gene beginning
with the start codon and terminating with the stop codon.
In addition, the sense and antisense primers were tailored
with the restriction sites for XbaI and BamHI respectively.
The product was gel-purified and digested with XbaI and
BamHI and then cloned into pTECH1 which had previously been
digested with these enzymes and subsequently gel-purified.

Expression of the TetC-P28 fusion protein

Expression of the TetC-P28 fusion protein was
evaluated by SDS-PAGE and Western blotting of bacterial
cells harbouring the construct  It was found that the
fusion protein remains soluble, cross-reacts with antisera
to both TetC and P28, and is also of the expected molecular
weight, 80kDal, for a full length fusion.

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The fusion protein was stably expressed in a number of different genetic backgrounds including E.coli (TG2) and S. typhimurium (SL5338,SL3261) as judged by SDS-PAGE and Western blotting. Of interest was a minor band of 50kDal which co-migrates with the TetC-Hinge protein alone and cross-reacts exclusively with the anti-TetC sera is visible in a Western blot. As the codon selection in the hinge region has been designed to be suboptimal, the rare codons may cause pauses during translation which may occasionally lead to the premature termination of translation, thus accounting for this band.

Affinity purification of the TetC-P28 fusion

Glutathione is the natural substrate for P28, a glutathione S-transferase. The amino acid residues involved in binding glutathione are thought to be spatially separated in the primary structure of the polypeptide and brought together to form a glutathione binding pocket in the tertiary structure (P. Reinemer et al. EMBO, J8, 1997-2005, 1991). In order to gauge whether the P28 component of the fusion has folded correctly to adopt a conformation capable of binding glutathione, its ability to be affinity purified on a glutathione-agarose matrix was tested. The results obtained (not shown) demonstrated that TetC-P28 can indeed bind to the matrix and the binding is reversible, as the fusion can be competitively eluted with free glutathione.

EXAMPLE 4

Construction of pTECH2-P28(aa115-131) peptide fusions

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Complementary oligonucleotides encoding the aa115-131 peptide were designed with a codon selection for optimal expression in E.coli [H. Grosjean et al idem]. The oligonucleotides were tailored with BglII and SpeI cohesive ends which were generated upon annealing and cloned into pTECH2 which had previously been digested with BamHI and SpeI (Figure 3).

Repeated tandem copies of the epitopes (repitopes) were constructed in pTECH2 by the following approach. The recombinant fusion vector was digested with XbaI and SpeI and to each digest was added a second restriction enzyme which cuts uniquely elsewhere within the vector, e.g. PstI which makes a cut exclusively within the ampicillin resistance gene (Figure 4). DNA fragments containing the epitope sequences can be purified from each of the double digests, mixed and then ligated. XbaI cleaves its target sequence to generate a 5'-CTAG overhang which is compatible with the SpeI overhang. Upon ligation the recognition sequences of both these enzymes are destroyed. In this way the XbaI-SpeI restriction sites remain unique and the procedure can be simply and effectively repeated to construct recombinant fusion vectors expressing four or eight tandem copies of the epitopes (Figure 4). A similar strategy has been used by others in the generation of a multimeric fusion protein for the production of a neuropeptide [T. Kempe et al. Gene 39, 239-45, 1985].

Expression of the TetC-peptide fusion proteins

Expression of the TetC-peptide fusions as monomeric,

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dimeric, tetrameric, and octameric tandem peptide repeats was evaluated by SDS-PAGE and Western blotting of the bacterial strains harbouring the constructs. The fusion proteins remain soluble, cross-react with both antisera to TetC and P28, and are also of the expected molecular weight [Figure 5]. Furthermore the fusion proteins are expressed in a number of different genetic backgrounds including E.coli (TG2) and S. typhimurium (SL5338, SL3261) as judged by SDS-PAGE and Western blotting. There appeared to be some degradation of the repitopes consisting of higher numbers of copies, as indicated by the appearance of faint bands of lower molecular weight seen in Western blots probed with the anti-P28 antibody.

The size of the bands suggested that they consisted of reduced copy number fusions to TetC. As was the case with the TetC-P28 fusion described above, the level of expression of the TetC-peptide fusions was less than that of TetC alone from pTECH2, with the expression level gradually decreasing with increasing copy number.

EXAMPLE 5

Immunological Studies

Stability of the plasmid constructs in vivo and immunisation of mice

BALB/c mice were given approx. $10^6$ cfu i/v or $5 \times 10^9$ orally of S. typhimurium SL3261 and SL3261 harbouring the different constructs. Viable counts on homogenates of liver, spleen and (for orally inoculated mice) lymph nodes performed from days 1-8 (epitope fusions) and 1-11 (vector,

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octamer and P28 fusions) were similar on media with and without ampicillin, indicating that the plasmids were not being lost during growth in the tissues.

## Antibody responses in mice immunised intravenously

### Antibody responses to the TetC-P28 fusion

Tail bleeds were taken weekly on weeks 3 to 6 from animals from each group of 8 mice. Figure 5 shows that in mice immunised with salmonellae expressing the TetC-P28 fusion, antibody responses to recombinant P28 appeared by week 3, and were positive in 6/6 mice from week 4 onwards. No anti-P28 antibodies were detected in sera from mice immunised with either SL3261 or SL3261-pTETnir15 or pTECH2.

All mice immunised with salmonellae expressing TetC, either alone or as the TetC-P28 fusion (but not with salmonellae alone), made antibody to TetC appearing as early as the third week. (Figure 6).

### Antibody responses to the TetC-peptide fusions

Mice immunised with salmonellae expressing TetC fused to multiple copies of the aa 115-131 peptide were bled as above and the sera tested by ELISA against the synthetic 115-131 peptide chemically conjugated to ovalbumin, and against recombinant P28. Figure 7 shows that antibody responses to the peptide were detected as early as week 3 and increased thereafter, with responses being stronger to fusions containing greater numbers of copies of the peptide. The octameric fusions elicited the best responses with 4-5 mice positive. No antibody responses were detected against ovalbumin-monomer or recombinant P28 in

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mice immunised either with SL3261, pTECH2 or the monomeric epitope fusion.

Some of the anti-epitope sera recognised the full length P28 protein in ELISA (Figure 5). One mouse injected with the dimeric fusion was positive at week 5, another mouse injected with the tetrameric fusion was positive at week 3. Thereafter sera from at least two mice injected with the octameric fusion consistently recognised P28 from week four up to week six.

In summary the antibody responses against the repitopes improved dramatically with increasing copy number, with the tetrameric and octameric repitope fusions being the most potent. No antibody responses to the monomeric fusion were detected.

Antibody response to TetC in mice immunised with the different fusions

The antibody response to TetC was not the same in all groups; the addition of C-terminal fusions to TetC clearly modified the response. Figure 6 shows that the antibody response to TetC elicited by the vector pTECH2 (TetC-Hinge alone) was significantly less than the TetC response to the parental vector, pTETnir15. Surprisingly, the addition to TetC of fusions of increasing size dramatically restores the response to TetC. The anti-TetC response to the largest fusion, full length P28 in pTECH1, was similar to the response to TetC obtained from the parental plasmid (under the conditions tested). Sera from mice injected with non-recombinant SL3261 did not react with TetC at any

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time during the period tested.


## Antibody responses in mice immunised orally

Groups of 10 mice were immunised orally with approx. $5 \times 10^9$ cfu of SL3261 alone or carrying pTECH1, or pTECH1-P28, given intragastrically in 0.2ml via a gavage tube. Bleeds taken from week 3 to week 10 showed that most mice receiving the recombinant salmonellae made antibody to TetC as early as week 3 (Figure 8). Mice immunised with the TetC-P28 fusion made antibody to P28 which was detectable in approximately half of the mice by week 8, and then declined (Figure 9).


## Antibody responses in mice immunised with the purified fusion protein

Mice were immunised subcutaneously with affinity purified TetC-P28 fusion protein adsorbed on aluminium hydroxide. Controls received commercial tetanus toxoid alone. Preliminary results indicate that animals given the fusion protein make an antibody response to both TetC and to P28 (data not shown). No anti-P28 antibody was detected in mice given tetanus toxoid.


## T-cell responses to TetC and P28

Mice were immunised i/v with approximately $10^6$ cfu of SL3261, SL3261(pTETnir15) and SL3261(pTECH1-P28). Six months later T-cell responses as IL-2/IL-4 production were measured against salmonella whole cell soluble extract,

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TetC, recombinant P28 and whole adult worm antigen as described in the section headed Materials and Methods below. Table 2 shows that cells from both groups produced an IL-2/IL-4 response to the sodium hydroxide treated salmonella extract and to TetC. However, cells from mice immunised with the salmonellae expressing the TetC-P28 fusion also responded to both recombinant P28 and whole worm extract.

Thus the salmonella delivery system has elicited both humoral and cellular (T-cell) immune responses to P28.

The salmonellae expressing the recombinant antigens all persisted in the mouse tissues as well as the parental strain, and the plasmids were not lost in vivo.

Constructs expressing higher molecular weight fusions (full length P28 and octamer) proved to be the most immunogenic. It may be that the immune response has been promoted by the carrier TetC providing additional T-cell helper epitopes [Francis et al. Nature 330: 168-170, 1987]. By week 4 all the mice immunised with cells carrying pTECH1-P28 responded to both TetC and also the full length P28 protein following i/v immunisation. Mice immunised orally also responded to TetC and P28, although not all the mice responded to P28. It may well be that the response to P28, could be improved by boosting. Improved constructs consisting of codon optimised hinge regions, codon optimised P28, and multiple copies of full length P28, are currently in preparation.

The antibody responses to the epitopes improved

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dramatically with increasing copy number, with the tetramer and octamer "repitope" fusions displaying the greatest potency.

EXAMPLE 6

Cloning of HPVE7 protein in pTECH2

The full-length HPV type 16 E7 protein gene was cloned into plasmid pTECH2 by an in frame insertion of the gene in the BamHI site of the vector hinge region.

The E7 gene was obtained from plasmid pGEX16E7 (S.A. Comerford et al. J Virology, 65, 4681-90 1991). The gene in this plasmid is flanked by two restriction sites: a 3' BamHI site and a 5' EcoRI site. pGEX16E7 DNA was digested with EcoRI and blunt ended by a filling up reaction using Sequenase (DNA polymerase from USB). It was then digested with BamHI to release the 0.3 Kbp full length E7 gene.

The gel purified gene was ligated to BamHI-EcoRV double digested pTECH2 and this ligation mixture used to transform competent E.coli HB101 bacteria.

Recombinant colonies were selected by colony blotting using two monoclonal antibodies against HPV16 E7 protein as probes, namely 6D and 4F (R.W. Tindle, et al J Gen.Vir. 71,1347-54 1990). One of these colonies, named pTE79, was chosen for further analysis.

Protein extracts from pTE79 transformed E.coli grown in both aerobic and anaerobic conditions were prepared and analysed by SDS-PAGE and Western blotting. Growth in anaerobic conditions resulted in expression of a recombinant molecule of about 60 KDal which reacted with

monoclonal antibodies 6D and 4F and a rabbit polyclonal
serum against Tetanus fragment C.

EXAMPLE 7

Construction of pTECH2-gD

An immunologically important antigen from herpes
simplex virus type 1 [HSV1] is glycoprotein D, termed gD1
(R.J. Watson *et al* Science 218, 381-383 1982). A truncated
gD1 gene cassette, lacking the transmembrane and
cytoplasmic domains aa26-340, was synthesised by PCR. The
PCR primers used are shown in Table 3. The forward primer
was designed to encode the N-terminus of the mature protein
and the reverse primer encoded the amino acids immediately
5' to the transmembrane domain. In addition the primers
were tailored with BamHI and SpeI restriction sites
respectively. The template for the PCR reation was the
plasmid pRWFG [a HSV1 gD BamHI-J clone from strain Patton
in pBR322; a kind gift from Dr. T. Minson, Cambridge
University]. The amplification product was digested with
BamHI and SpeI and cloned into pTECH2 which had previously
been digested with the respective enzymes.

Expression of the TetC-gD1 fusion protein was assessed
by SDS-PAGE and Western blotting of bacterial strains
harbouring the constructs. The Western blots were probed
with either anti-TetC polyclonal sera or a monoclonal
anitbody directed against amino acids 11-19 of the mature
gD [designated LP16, obtained from Dr. T. Minson,
Cambridge]. The fusion protein is expressed as a 85kDal
band visible on Western blots together with lower molecular

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weight bands down to 50kDal in size. The lower molecular weight bands could correspond to proteolytic cleavage products of gD or represent the products of premature translational termination within the coding region of gD due to ribosomal pausing. The fusion protein is expressed in the salmonella strains SL5338 and SL3261.

EXAMPLE 8

Construction of pTECH2- FMDV/SIV Repitopes

Peptides from the foot and mouth disease virus (FMDV; serotype A12] viral protein1 [VP1; aa136-159] and the V2 loop from simian immunodeficiency virus [SIV] envelope protein [gp120; aa171-190] were cloned into pTECH2 (M.P. Broekhuijsen et al J. Gen. Virol. 68, 3137-45 1987; K.A. Kent et al. AIDS Res. and Human Retro. 8:1147-1151 1992].

Complementary oligonucleotides encoding the peptides were designed with a codon selection for optimal expression in E. coli [H. Grosjean et al Gene, 18, 199-209, 1982]. The oligonucleotides are shown in Table 3. The oligonucleotides were tailored with BglII and SpeI cohesive ends which were generated upon annealing and cloned into pTECH2 which had previously been digested with BamHI and SpeI (Figure 3). Dimeric, tetrameric and octameric fusions of these peptides were constructed as described previously.

Expression of the TetC-fusions was assessed by SDS-PAGE and Western blotting with a polyclonal sera directed against TetC and monoclonal antibodies directed against either the FMDV or the SIV epitopes. The FMDV and SIV

repitope constructs expressed the TetC fusion proteins in both SL5338 and SL3261.

EXAMPLE 9

Construction of pTECH2- gp120-P28 Peptide Heteromers

To explore the possibility of delivering more than one type of epitope from a single molecule of TetC, fusions have been made with the P28 and SIV repitopes to produce a tripartite protein. This form of construction has been facilitated by the modular nature of the vector which allows the assembly of vector modules containing different repitopes. These "heteromers" express either tandem dimers or tetramers of the P28 and SIV repitopes. To investigate the effect of the position of a particular repitope in the TetC-Repitope A-Repitope B fusion on its expression level, stability, and immunogenicity, the converse combinations have also been constructed i.e. TetC-Repitope B-Repitope A, as is shown in Figure 11. "Heteromers" constructed in this way are TetC-P28 dimer-SIV dimer, TetC-SIV dimer-P28 dimer, TetC-P28 tetramer-SIV tetramer and TetC-SIV tetramer-P28 tetramer.

Expression of the tripartite fusions were evaluated by SDS-PAGE and Western blotting using the antibody reagents described above. These heteromer constructs are all expressed in the *Salmonella* strains SL5338 and SL3261, but intriguingly the expression level and stability is greater in one dimer-dimer and tetramer-tetramer combination (TetC-gp120-P28] than the converse.

EXAMPLE 10

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## MATERIALS AND METHODS

<u>Plasmids, Oligonucleotides, and the Polymerase Chain Reaction</u>

The plasmid pTETnir15 directs the expression of fragment C from tetanus toxin under the control of the <u>nirB</u> promoter [Chatfield *et al.* <u>idem</u> Oxer *et al.* <u>idem</u>

The TetC-hinge fusion vector pTECH1 was constructed from pTETnir15 by the polymerase chain reaction (PCR) described by Mullis *et al*, 1986. PCR was performed using the high-fidelity thermostable DNA polymerase from <u>Pyrococcus furiosus</u>, which possesses an associated 3'-5' exonuclease proofreading activity [K.S. Lundberg *et al* Gene 108: 1-6, 1991]. The amplification reaction was performed according to the manufacturer's instructions (Stratagene).

## <u>Bacterial Strains</u>

The bacterial strains used were <u>E.coli</u> TG2 (recA; [J. Sambrook *et al.* Molecular cloning: a laboratory manual. Cold Spring Harbor, New York, 1989]). <u>S.typhimurium</u> SL5338 (<u>galE</u> r⁻m⁺ [A. Brown J. Infect. Dis. 155: 86-92, *et al* J. Infect. Dis. 155: 86-92, 1987]) and SL3261 (<u>aroA</u>); [S.K. Hoiseth *et al* Nature 291, 238-9, 1981]. Bacteria were cultured in either L or YT broth and on L-agar with ampicillin (50 µg/ml) if appropriate. Plasmid DNA prepared in <u>E.coli</u> was first modified by transformation into SL5338 to increase the efficiency of electroporation into the SL3261 <u>aroA</u> (r⁺m)vaccine. For electroporation, cells growing in mid-log phase were harvested and washed in half the initial culture volume of ice-cold water, 1/10 volume

of ice-cold glycerol (10%), and finally the cells were resuspended to a concentration of $10^{10}$ cells/ml in ice-cold glycerol (10%). To a pre-chilled cuvette was added a mix of 60 μl cells and 100 ng of plasmid DNA. The cells were pulsed using the Porator from Invitrogen ( settings: voltage=1750 μv, capacitance = 40 μF, resistance = 500). Prewarmed L-broth supplemented with 20 mM glucose was added immediately and the cells grown at 37°C with gentle shaking for 1-1.5 h. The cells were than plated on L-agar plates containing ampicillin and incubated at 37°C for 16 h.

## SDS-PAGE and Western Blotting

Expression of the TetC fusions was tested by SDS-PAGE and Western blotting. Cells growing in mid-log phase with antibiotic selection were harvested by centrifugation and the proteins fractionated by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane by electroblotting and reacted with either a polyclonal rabbit antiserum directed against TetC or the full length P28 protein. The blots were then probed with goat anti-rabbit-Ig conjugated to horseradish peroxidase (Dako, UK) and developed with 4-chloro-1-naphthol.

## Glutathione-Agarose Affinity Purification

Bacterial cells expressing the TetC full length P28 gene fusion were grown to log phase, chilled on ice, and harvested by centrifugation at 2500Xg for 15 min at 4°C. The cells were resuspended in 1/15th the original volume of ice-cold phosphate buffered saline (PBS) and lysed by sonication in a MSE Soniprep. The insoluble material was

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removed by centrifugation and to the supernatant was added 1/6 volume of a 50% slurry of pre-swollen glutathione-agarose beads. (Sigma, UK.). After mixing gently at room temperature for 1 h the beads were collected by centrifugation at 1000Xg for 10 sec. The supernatant was discarded and the beads resuspended in 20 volumes of cold PBS-0.5% Triton X-100 and the beads collected again by centrifugation. The washing step was repeated three more times. The fusion protein was eluted by adding 1 volume of 50 mM Tris-HCl, pH 8.0 containing 5.0 mM reduced glutathione (Sigma). After mixing gently for 10 min the beads were pelleted as before and the supernatant removed. The elution step was repeated five more times and the supernatant fractions analysed by SDS-PAGE.

Animals

Female BALB/c mice were purchased from Harlan Olac UK Blackthorn, Bicester, UK, and used when at least 8 weeks of age.

Inoculations and viable counting or organ homogenates

Bacteria were grown in tryptic soy broth (Oxoid) supplemented with 100 μg/ml ampicillin as required. For intravenous inoculation, stationary cultures were diluted in PBS and animals were given approx. $10^5$ cfu in a lateral tail vein in 0.2 ml. For oral inoculation, bacteria were grown in shaken overnight cultures, concentrated by centrifugation, and animals received approximately $5 \times 10^9$ cfu in 0.2 ml intragastrically via a gavage tube. The inoculum doses were checked by viable counts on tryptic soy agar.

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For viable counts on organ homogenates, groups of 3 mice were sacrificed at intervals, the livers and spleen and (for orally inoculated mice) a pool of mesenteric lymph nodes were homogenised separately in 10 ml distilled water in a Colworth stomacher [C.E. Hormaeche Immunology 37, 311-318, 1979] and viable counts performed on tryptic soy agar supplemented with 100 µg/ml ampicillin.

Measurement of antibody responses

Antibodies were measured by solid phase immunoassay. 96-well-flat bottomed plates were coated with either 0.1 µg of TetC (a kind gift from Dr N Fairweather, the Wellcome Foundation, Beckenham UK) or 1 µg of recombinant P28 (a kind gift from Dr R Pierce, Pasteur Institute, Lille, France) in 100 µl of 0.1 M carbonate buffer, pH 9.6. After overnight incubation at 4°C the plates were incubated for 1 h at 37°C. Blocking of non-specific binding sites was carried out by incubation with 200 µl of 2% casein (BDH, Poole, UK) in PBS pH 7.0 for 1 h at 37°C. Plates were washed three times with 0.05% Tween 20 (Sigma) in PBS with a semiautomatic ELISA washer (Titertek, Flow/ICN, Herts UK). 100 µl of sera from inoculated mice diluted 1:20 in 2% casein was added to each well and the plates were incubated for one hour at 37°C. The plates were washed as above and 100 µl of horse radish peroxidase conjugated goat antimouse immunoglobulins (Dako, Bucks UK), diluted according to the manufacturer's instructions in 2% casein in PBS, was added to each well and incubated for one hour at 37°C. The plates were washed as above and three more

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washes were given with PBS alone. The plates were developed using 3,3',3,3'-tetramethylbenzidine dihydrochloride (Sigma) according to the manufacturer's instructions using phosphate/citrate buffer, pH 5.0 and 0.02% hydrogen peroxide. The plates were incubated for 10-15 min at 37°C after which the reaction was stopped with 25 μl 3M $H_2SO_4$ (BDH). The plates were read in an ELISA reader at 450 nm.

Measurement of T-cell responses

Spleens from mice vaccinated 6 months in advance were removed aseptically and single cell suspensions were prepared by mashing the spleens through a stainless steel sieve with the help of a plastic plunger. Cells were washed once in RPMI1640 medium (Flow/ICN) at 300xg and incubated in Gey's solution to lyse the red cells. White cells were washed twice more as above and resuspended in complete medium, i.e. RPMI1640 supplemented with 100 U/ml penicillin G (Flow/ICN), 100 μg/ml streptomycin (Flow/ICN), $2 \times 10^{-5}$M B-mercapto-ethanol (Sigma), 1mM N-(2-hydroxyethyl-piperazine-N'-(2-ethanesulphonic acid) (HEPES) (Flow/ICN) and 10% heat inactivated newborn bovine serum (Northumbria Biolabs, Northumberland, UK). For isolation of T-cells, spleen cells were treated as above and after lysis of red cells the white cells were resuspended in warm (37°C) RPMI1640 and passed through a Wigzell glass bead column [H. Wigzell, et al Scand. J. Immunol 1: 75-87, 1972].

Cells were plated at $2 \times 10^6$/ml in a final volume of 200 μl of complete medium in 96-well plates in the presence of

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the relevant antigens. These were either an alkali-treated whole cell soluble extract of S.typhimurium C5 prepared as described in Villarreal *et al.* [Microbial Pathogenesis 13: 305-315, 1992] at 20 µg/ml final concentration; TetC at 10 µg/ml; recombinant Schistosoma mansoni P28 at 50 µg/ml; and S. mansoni whole adult worm extract (a kind gift from Dr D Dunne, Cambridge University) at 20 µg/ml. Cells were incubated in a 95% humidity, 5% $CO_2$, 37°C atmosphere.

Feeder cells for T-cells for animals immunised with SL3261(pTECH1-P28) were obtained from syngeneic BALB/c naive spleens prepared as above. For mice immunised with pTETnir15, feeder cells were obtained from similarly immunised animals. After red cell lysis and two washes with RPM11640 cells were X-ray irradiated at 2000 rads and washed twice more. These antigen presenting cells were resuspended in complete medium to give a final ratio of 1:1 with T-cells.

IL-2 production and assay

T-cell suspensions were plated as above. After two days, 50 µl of supernatant was harvested and added to $1 \times 10^4$ cells/well CTLL-2(IL-2 dependent) in 50 µl of medium. CTLL-2 cells were obtained from Dr J Ellis, University College, London UK and maintained in RPMI1640 supplemented as above, substituting the newborn bovine serum for foetal bovine serum. After 20 h, 20 µl of MTT at a concentration of 5 mg/ml in PBS were added. MTT transformation was measured as indicated elsewhere [Tada *et al.* J. Immunol. Methods 93: 157-165, 1986]. results were expressed as the

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mean of the optical density of triplicates read at 570 nm using a reference filter of 630 nm. Significance was determined by Student's t-test.


## BACTERIAL SAMPLE DEPOSITS

Salmonella typhimurium strains SL3261-pTECH1, SL3261-pTECH1-P28, SL3261-pTECH2, SL3261-pTECH2-P28 Octamer and PTE79 have been deposited at the National Collection of Type Cultures, 61 Colindale Avenue, London, NW9 5HT, UK, on 15th July 1993 under Deposit Numbers NCTC 12831, NCTC 12833, 12832, 12834 and 12837 respectively.

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## TABLE 1


DNA SEQUENCES OF OLIGONUCLEOTIDES UTILISED IN THE
CONSTRUCTION OF THE TETC-HINGE VECTORS


A). Primer 1. Sense PCR (21mer).    (SEQ ID NO: 4)



                    SacII
    5'**AAA** GAC TCC **GCG GGC** GAA GTT -3'
        TETANUS TOXIN C FRAGMENT SEQ.



B).Primer 2. Anti-Sense PCR Primer (64mer).    (SEQ ID NO: 5)



         BamHI    STOP    SpeI       XbaI     HINGE REGION
    5'- CTAT **GGA TCC** <u>TTA</u> **ACT AGT** GAT TCT AGA GGG CCC CGG CCC

        GTC GTT GGT CCA ACC TTC ATC GGT -3'
        TETANUS TOXIN C FRAGMENT SEQ. 3'-END


C). The pTECH2 Linker   (SEQ ID NO: 6)


    XbaI   BamHI  EcoRV  HindIII SpeI  Stop  *XBamHI**
  5'-CTAGA GGATCC GATATC AAGCTT ACTAGT TAA T-3'
    3'-T CCTAGG CTATAG TTCGAA TGATCA ATT ACTAG-5'


  *This BamHI recognition sequence is now destroyed.

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## TABLE 2

T-Cell responses (IL-2/IL-4 production) elicited by alkali treated salmonella whole cell extract (C5NaOH), TetC, *Schistosoma mansoni* whole adult worm antigen (SWA), and recombinant P28 in mice immunised with SL3261(pTETnir15) or SL3261(pTECH1.P28).

| Immunising strain | Stimulating antigen | | | | |
|---|---|---|---|---|---|
| | none | C5NaOH | TetC | P28 | SWA |
| SL3261 (pTETnir15) | 2±4 | 67±5 | 41±1 | 0 | 0 |
| SL3261 (pTECH1-P28) | 6±2.6 | 109±10 | 50±8 | 25±8 $p<0.001$ | 17±6 $p<0.01$ |

Results expressed as $(A_{570}-A_{630})$ x 1000±S.D.

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## TABLE 3

Ogligonucleotide Sequences for HSV, FMDV, and SIV.

### HSV1 gD Gene

PCR Primer 1: 5'-AATGGATCCAAATATGCCCTGGCGGATGC-3'
(SEQ ID NO: 7)

PCR Primer 2: 5'-TTAACTAGTGTTGTTCGGGGTGGCCGGGGGAT-3'
(SEQ ID NO: 8)

### FMDV VP1 Epitope

Oligo 1:
5'-GATCTAAATACTCTGCTTCTGGTTCTGGTGTTCGTGGTGAC
TTCGGTTCTCTGGCTCCGCGTGTTGCTCGTCAGCTGA-3'
(SEQ ID NO: 9)

Oligo 2:
5'-CTAGTCAGCTGACGAGCAACACGCGGAGCCAGAGAACCGAA
GTCACCACGAACACCAGAACCAGAAGCAGAGTATTTA-3
(SEQ ID NO: 10)

### SIV gp120 Epitope

Oligo 1:
5'-GATCTAACATGACCGGTCTGAAACGTGATAAAACCAAAGAA
TACAACGAAACCTGGTACTCTACCA-3'
(SEQ ID NO: 11)

Oligo 2:
5'-CTAGTGGTAGAGTACCAGGTTTCGTTGTATTCTTTGGTTTT
ATCACGTTTCAGACCGGTCATGTTA-3'
(SEQ ID NO: 12)

### Sm P28 Gene

PCR Primer 1: 5'-TAGTCTAGAATGGCTGGCGAGCATATCAAG-3'
(SEQ ID NO: 13)

PCR Primer 2: 5'-TTAGGATCCTTAGAAGGGAGTTGCAGGCCT-3'
(SEQ ID NO: 14)

### Sm P28 Epitope

Oligo 1:
5'-GATCTAAACCGCAGGAAGAAAAAGAAAAAATCACCAAAGAAA
TCCTGAACGGCAAAA-3'
(SEQ ID NO: 15)

Oligo 2:
5'-CTAGTTTTGCCGTTCAGGATTTCTTTGGTGATTTTTTCTTTTTCT
TCCTGCGGTTTA-3'
(SEQ ID NO: 16)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
       (A) NAME: MEDEVA HOLDINGS BV
       (B) STREET: CHURCHILL-LAAN 223
       (C) CITY: AMSTERDAM
       (E) COUNTRY: THE NETHERLANDS
       (F) POSTAL CODE (ZIP): 1078 ED

    (ii) TITLE OF INVENTION: VACCINES

    (iii) NUMBER OF SEQUENCES: 20

    (iv) COMPUTER READABLE FORM:
       (A) MEDIUM TYPE: Floppy disk
       (B) COMPUTER: IBM PC compatible
       (C) OPERATING SYSTEM: PC-DOS/MS-DOS
       (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

    (vi) PRIOR APPLICATION DATA:
       (A) APPLICATION NUMBER: GB 9216317.9
       (B) FILING DATE: 31-JUL-1992

    (vi) PRIOR APPLICATION DATA:
       (A) APPLICATION NUMBER: GB 9306398.0
       (B) FILING DATE: 26-MAR-1993


(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 68 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iii) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
       (A) ORGANISM: Escherichia coli

    (ix) FEATURE:
       (A) NAME/KEY: promoter
       (B) LOCATION: 1..61


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AATTCAGGTA AATTTGATGT ACATCAAATG GTACCCCTTG CTGAATCGTT AAGGTAGGCG       60

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GTAGGGCC 68

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 68 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iii) ANTI-SENSE: NO


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AATTCAGGTA AATTTGATGT ACATCAAATG GTACCCCTTG CTGAATCGTT AAGGTAGGCG 60

GTAGGGCC 68

(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 60 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iii) ANTI-SENSE: NO


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTACCGCCTA CCTTAACGAT TCAGCAAGGG GTACCATTTG ATGTACATCA AATTTACCTG 60

(2) INFORMATION FOR SEQ ID NO: 4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

44

(iii) ANTI-SENSE: NO


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AAAGACTCCG CGGGCGAAGT T                                          21

(2) INFORMATION FOR SEQ ID NO: 5:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 64 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iii) ANTI-SENSE: YES


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTATGGATCC TTAACTAGTG ATTCTAGAGG GCCCCGGCCC GTCGTTGGTC CAACCTTCAT     60

CGGT                                                             64

(2) INFORMATION FOR SEQ ID NO: 6:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 33 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: double
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iii) ANTI-SENSE: NO


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTAGAGGATC CGATATCAAG CTTACTAGTT AAT                              33

(2) INFORMATION FOR SEQ ID NO: 7:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 29 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single

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        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iii) ANTI-SENSE: NO



   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AATGGATCCA AATATGCCCT GGCGGATGC                                    29

(2) INFORMATION FOR SEQ ID NO: 8:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 31 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iii) ANTI-SENSE: NO



   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TAACTAGTGT TGTTCGGGGT GGCCGGGGGA T                                 31

(2) INFORMATION FOR SEQ ID NO: 9:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 78 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iii) ANTI-SENSE: NO



   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GATCTAAATA CTCTGCTTCT GGTTCTGGTG TTCGTGGTGA CTTCGGTTCT CTGGCTCCGC   60

GTGTTGCTCG TCAGCTGA                                                78
```

(2) INFORMATION FOR SEQ ID NO: 10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 78 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

  (iii) HYPOTHETICAL: NO

  (iii) ANTI-SENSE: NO


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CTAGTCAGCT GACGAGCAAC ACGCGGAGCC AGAGAACCGA AGTCACCACG AACACCAGAA     60

CCAGAAGCAG AGTATTTA     78

(2) INFORMATION FOR SEQ ID NO: 11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 66 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

  (iii) HYPOTHETICAL: NO

  (iii) ANTI-SENSE: NO


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GATCTAACAT GACCGGTCTG AAACGTGATA AAACCAAAGA ATACAACGAA ACCTGGTACT     60

CTACCA     66

(2) INFORMATION FOR SEQ ID NO: 12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 66 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

  (iii) HYPOTHETICAL: NO

        (iii) ANTI-SENSE: NO


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CTAGTGGTAG AGTACCAGGT TTCGTTGTAT TCTTTGGTTT TATCACGTTT CAGACCGGTC        60

ATGTTA                                                                   66

(2) INFORMATION FOR SEQ ID NO: 13:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iii) ANTI-SENSE: NO


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TAGTCTAGAA TGGCTGGCGA GCATATCAAG                                         30

(2) INFORMATION FOR SEQ ID NO: 14:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iii) ANTI-SENSE: NO


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TTAGGATCCT TAGAAGGGAG TTGCAGGCCT                                         30

(2) INFORMATION FOR SEQ ID NO: 15:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 57 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single

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            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

      (iii) HYPOTHETICAL: NO

      (iii) ANTI-SENSE: NO


       (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GATCTAAACC GCAGGAAGAA AAAGAAAAAA TCACCAAAGA AATCCTGAAC GGCAAAA              57

(2) INFORMATION FOR SEQ ID NO: 16:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 57 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

      (iii) HYPOTHETICAL: NO

      (iii) ANTI-SENSE: NO


       (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CTAGTTTTGC CGTTCAGGAT TTCTTTGGTG ATTTTTTCTT TTTCTTCCTG CGGTTTA              57

(2) INFORMATION FOR SEQ ID NO: 17:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 3754 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: double
              (D) TOPOLOGY: circular

       (ii) MOLECULE TYPE: DNA (genomic)

      (iii) HYPOTHETICAL: NO

      (iii) ANTI-SENSE: NO


       (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTCAGGTAAA TTTGATGTAC ATCAAATGGT ACCCCTTGCT GAATCGTTAA GGTAGGCGGT          60

AGGGCCCAGA TCTTAATCAT CCACAGGAGA CTTTCTGATG AAAAACCTTG ATTGTTGGGT          120

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CGACAACGAA GAAGACATCG ATGTTATCCT GAAAAAGTCT ACCATTCTGA ACTTGGACAT        180

CAACAACGAT ATTATCTCCG ACATCTCTGG TTTCAACTCC TCTGTTATCA CATATCCAGA        240

TGCTCAATTG GTGCCGGGCA TCAACGGCAA AGCTATCCAC CTGGTTAACA ACGAATCTTC        300

TGAAGTTATC GTGCACAAGG CCATGGACAT CGAATACAAC GACATGTTCA ACAACTTCAC        360

CGTTAGCTTC TGGCTGCGCG TTCCGAAAGT TTCTGCTTCC CACCTGGAAC AGTACGGCAC        420

TAACGAGTAC TCCATCATCA GCTCTATGAA GAAACACTCC CTGTCCATCG GCTCTGGTTG        480

GTCTGTTTCC CTGAAGGGTA ACAACCTGAT CTGGACTCTG AAAGACTCCG CGGGCGAAGT        540

TCGTCAGATC ACTTTCCGCG ACCTGCCGGA CAAGTTCAAC GCGTACCTGG CTAACAAATG        600

GGTTTTCATC ACTATCACTA ACGATCGTCT GTCTTCTGCT AACCTGTACA TCAACGGCGT        660

TCTGATGGGC TCCGCTGAAA TCACTGGTCT GGGCGCTATC CGTGAGGACA ACAACATCAC        720

TCTTAAGCTG GACCGTTGCA ACAACAACAA CCAGTACGTA TCCATCGACA AGTTCCGTAT        780

CTTCTGCAAA GCACTGAACC CGAAAGAGAT CGAAAAACTG TATACCAGCT ACCTGTCTAT        840

CACCTTCCTG CGTGACTTCT GGGGTAACCC GCTGCGTTAC GACACCGAAT ATTACCTGAT        900

CCCGGTAGCT TCTAGCTCTA AAGACGTTCA GCTGAAAAAC ATCACTGACT ACATGTACCT        960

GACCAACGCG CCGTCCTACA CTAACGGTAA ACTGAACATC TACTACCGAC GTCTGTACAA       1020

CGGCCTGAAA TTCATCATCA AACGCTACAC TCCGAACAAC GAAATCGATT CTTTCGTTAA       1080

ATCTGGTGAC TTCATCAAAC TGTACGTTTC TTACAACAAC AACGAACACA TCGTTGGTTA       1140

CCCGAAAGAC GGTAACGCTT TCAACAACCT GGACAGAATT CTGCGTGTTG GTTACAACGC       1200

TCCGGGTATC CCGCTGTACA AAAAAATGGA AGCTGTTAAA CTGCGTGACC TGAAAACCTA       1260

CTCTGTTCAG CTGAAACTGT ACGACGACAA AAACGCTTCT CTGGGTCTGG TTGGTACCCA       1320

CAACGGTCAG ATCGGTAACG ACCCGAACCG TGACATCCTG ATCGCTTCTA ACTGGTACTT       1380

CAACCACCTG AAAGACAAAA TCCTGGGTTG CGACTGGTAC TTCGTTCCGA CCGATGAAGG       1440

TTGGACCAAC GACGGGCCGG GGCCCTCTAG AATCACTAGT TAAGGATCCG CTAGCCCGCC       1500

TAATGAGCGG GCTTTTTTTT CTCGGGCAGC GTTGGGTCCT GGCCACGGGT GCGCATGATC       1560

GTGCTCCTGT CGTTGAGGAC CCGGCTAGGC TGGCGGGGTT GCCTTACTGG TTAGCAGAAT       1620

GAATCACCGA TACGCGAGCG AACGTGAAGC GACTGCTGCT GCAAAACGTC TGCGACCTGA       1680

GCAACAACAT GAATGGTCTT CGGTTTCCGT GTTTCGTAAA GTCTGGAAAC GCGGAAGTCA       1740

GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC       1800
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GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG ATAACGCAGG      1860

AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT      1920

GGCGTTTTTC CATAGGCTCC GCCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA      1980

GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT      2040

CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC      2100

GGGAAGCGTG GCGCTTTCTC AATGCTCACG CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT      2160

TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG CCCGACCGCT GCGCCTTATC      2220

CGGTAACTAT CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC      2280

CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG      2340

GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC      2400

AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA CCGCTGGTAG      2460

CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA AAAAAAGGAT CTCAAGAAGA      2520

TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT      2580

TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG      2640

TTTTAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT      2700

CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTCGTTCA TCCATAGTTG CCTGACTCCC      2760

CGTCGTGTAG ATAACTACGA TACGGGAGGG CTTACCATCT GGCCCCAGTG CTGCAATGAT      2820

ACCGCGAGAC CCACGCTCAC CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGGAAG      2880

GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG      2940

CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC      3000

TGCAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA      3060

ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG      3120

TCCTCCGATC GTTGTCAGAA GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC      3180

ACTGCATAAT TCTCTTACTG TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA      3240

CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC      3300

AACACGGGAT AATACCGCGC CACATAGCAG AACTTTAAAA GTGCTCATCA TTGGAAAACG      3360

TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC      3420

CACTCGTGCA CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC      3480
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AAAAACAGGA AGGCAAAATG CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT    3540

ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG    3600

CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA GGGGTTCCGC GCACATTTCC    3660

CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC ATGACATTAA CCTATAAAAA    3720

TAGGCGTATC ACGAGGCCCT TTCGTCTTCA AGAA                                3754
```

(2) INFORMATION FOR SEQ ID NO: 18:

  (i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 3769 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: double
   (D) TOPOLOGY: circular

  (ii) MOLECULE TYPE: DNA (genomic)

  (iii) HYPOTHETICAL: NO

  (iii) ANTI-SENSE: NO


  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

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TTCAGGTAAA TTTGATGTAC ATCAAATGGT ACCCCTTGCT GAATCGTTAA GGTAGGCGGT     60

AGGGCCCAGA TCTTAATCAT CCACAGGAGA CTTTCTGATG AAAAACCTTG ATTGTTGGGT    120

CGACAACGAA GAAGACATCG ATGTTATCCT GAAAAAGTCT ACCATTCTGA ACTTGGACAT    180

CAACAACGAT ATTATCTCCG ACATCTCTGG TTTCAACTCC TCTGTTATCA CATATCCAGA    240

TGCTCAATTG GTGCCGGGCA TCAACGGCAA AGCTATCCAC CTGGTTAACA ACGAATCTTC    300

TGAAGTTATC GTGCACAAGG CCATGGACAT CGAATACAAC GACATGTTCA ACAACTTCAC    360

CGTTAGCTTC TGGCTGCGCG TTCCGAAAGT TTCTGCTTCC CACCTGGAAC AGTACGGCAC    420

TAACGAGTAC TCCATCATCA GCTCTATGAA GAAACACTCC CTGTCCATCG GCTCTGGTTG    480

GTCTGTTTCC CTGAAGGGTA ACAACCTGAT CTGGACTCTG AAAGACTCCG CGGGCGAAGT    540

TCGTCAGATC ACTTTCCGCG ACCTGCCGGA CAAGTTCAAC GCGTACCTGG CTAACAAATG    600

GGTTTTCATC ACTATCACTA CGATCGTCT GTCTTCTGCT AACCTGTACA TCAACGGCGT    660

TCTGATGGGC TCCGCTGAAA TCACTGGTCT GGGCGCTATC CGTGAGGACA ACAACATCAC    720

TCTTAAGCTG GACCGTTGCA ACAACAACAA CCAGTACGTA TCCATCGACA AGTTCCGTAT    780

CTTCTGCAAA GCACTGAACC CGAAAGAGAT CGAAAAACTG TATACCAGCT ACCTGTCTAT    840
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CACCTTCCTG CGTGACTTCT GGGGTAACCC GCTGCGTTAC GACACCGAAT ATTACCTGAT        900

CCCGGTAGCT TCTAGCTCTA AAGACGTTCA GCTGAAAAAC ATCACTGACT ACATGTACCT        960

GACCAACGCG CCGTCCTACA CTAACGGTAA ACTGAACATC TACTACCGAC GTCTGTACAA       1020

CGGCCTGAAA TTCATCATCA AACGCTACAC TCCGAACAAC GAAATCGATT CTTTCGTTAA       1080

ATCTGGTGAC TTCATCAAAC TGTACGTTTC TTACAACAAC AACGAACACA TCGTTGGTTA       1140

CCCGAAAGAC GGTAACGCTT TCAACAACCT GGACAGAATT CTGCGTGTTG GTTACAACGC       1200

TCCGGGTATC CCGCTGTACA AAAAAATGGA AGCTGTTAAA CTGCGTGACC TGAAAACCTA       1260

CTCTGTTCAG CTGAAACTGT ACGACGACAA AAACGCTTCT CTGGGTCTGG TTGGTACCCA       1320

CAACGGTCAG ATCGGTAACG ACCCGAACCG TGACATCCTG ATCGCTTCTA ACTGGTACTT       1380

CAACCACCTG AAAGACAAAA TCCTGGGTTG CGACTGGTAC TTCGTTCCGA CCGATGAAGG       1440

TTGGACCAAC GACGGGCCGG GGCCCTCTAG AGGATCCGAT ATCAAGCTTA CTAGTTAATG       1500

ATCCGCTAGC CCGCCTAATG AGCGGGCTTT TTTTTCTCGG GCAGCGTTGG GTCCTGGCCA       1560

CGGGTGCGCA TGATCGTGCT CCTGTCGTTG AGGACCCGGC TAGGCTGGCG GGGTTGCCTT       1620

ACTGGTTAGC AGAATGAATC ACCGATACGC GAGCGAACGT GAAGCGACTG CTGCTGCAAA       1680

ACGTCTGCGA CCTGAGCAAC AACATGAATG GTCTTCGGTT TCCGTGTTTC GTAAAGTCTG       1740

GAAACGCGGA AGTCAGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT       1800

TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT CCACAGAATC       1860

AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA       1920

AAAGGCCGCG TTGCTGGCGT TTTTCCATAG GCTCCGCCCC CCTGACGAGC ATCACAAAAA       1980

TCGACGCTCA AGTCAGAGGT GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC       2040

CCCTGGAAGC TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC       2100

CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCAATGC TCACGCTGTA GGTATCTCAG       2160

TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC GAACCCCCCG TTCAGCCCGA       2220

CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC CCGGTAAGAC ACGACTTATC       2280

GCCACTGGCA GCAGCCACTG GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC       2340

AGAGTTCTTG AAGTGGTGGC CTAACTACGG CTACACTAGA AGGACAGTAT TTGGTATCTG       2400

CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT CCGGCAAACA       2460

AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG CAGATTACGC GCAGAAAAAA       2520
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AGGATCTCAA GAAGATCCTT TGATCTTTTC TACGGGGTCT GACGCTCAGT GGAACGAAAA      2580

CTCACGTTAA GGGATTTTGG TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT      2640

AAATTAAAAA TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG      2700

TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC GTTCATCCAT      2760

AGTTGCCTGA CTCCCCGTCG TGTAGATAAC TACGATACGG GAGGGCTTAC CATCTGGCCC      2820

CAGTGCTGCA ATGATACCGC GAGACCCACG CTCACCGGCT CCAGATTTAT CAGCAATAAA      2880

CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG TGGTCCTGCA ACTTTATCCG CCTCCATCCA      2940

GTCTATTAAT TGTTGCCGGG AAGCTAGAGT AAGTAGTTCG CCAGTTAATA GTTTGCGCAA      3000

CGTTGTTGCC ATTGCTGCAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA TGGCTTCATT      3060

CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC CCCATGTTGT GCAAAAAAGC      3120

GGTTAGCTCC TTCGGTCCTC CGATCGTTGT CAGAAGTAAG TTGGCCGCAG TGTTATCACT      3180

CATGGTTATG GCAGCACTGC ATAATTCTCT TACTGTCATG CCATCCGTAA GATGCTTTTC      3240

TGTGACTGGT GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC GACCGAGTTG      3300

CTCTTGCCCG GCGTCAACAC GGGATAATAC CGCGCCACAT AGCAGAACTT TAAAAGTGCT      3360

CATCATTGGA AAACGTTCTT CGGGGCGAAA ACTCTCAAGG ATCTTACCGC TGTTGAGATC      3420

CAGTTCGATG TAACCCACTC GTGCACCCAA CTGATCTTCA GCATCTTTTA CTTTCACCAG      3480

CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC      3540

ACGGAAATGT TGAATACTCA TACTCTTCCT TTTTCAATAT TATTGAAGCA TTTATCAGGG      3600

TTATTGTCTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC AAATAGGGGT      3660

TCCGCGCACA TTTCCCCGAA AAGTGCCACC TGACGTCTAA GAAACCATTA TTATCATGAC      3720

ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT CTTCAAGAA              3769
```

(2) INFORMATION FOR SEQ ID NO: 19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: circular

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iii) ANTI-SENSE: NO

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(v) FRAGMENT TYPE: internal


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TCTAGAGGAT CCGATATCAA GCTTACTAGT TAATGATC                                       38

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 14 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Gly Pro Gly Pro Ser Arg Gly Ser Asp Ile Lys Leu Thr Ser
1              5                  10

## CLAIMS

1. A DNA construct comprising a promoter sequence operably linked to a DNA sequence encoding first and second proteins linked by a hinge region, characterised in that the promoter sequence is one having activity which is induced in response to a change in the surrounding environment.

2. A DNA construct according to claim 1 wherein the proteins are polypeptide immunogens.

3. A DNA construct according to claim 1 wherein the first heterologous protein is an antigenic sequence comprising tetanus toxin fragment C or epitopes thereof.

4. A DNA construct comprising a promoter sequence operably linked to a DNA sequence encoding linked first and second heterologous proteins wherein the first heterologous protein is an antigenic sequence comprising tetanus toxin fragment C or one or more epitopes thereof.

5. A DNA construct according to Claim 4 wherein the promoter sequence is one having activity which is induced in response to a change in surrounding environment.

6. A DNA construct according to any one of the preceding Claims wherein the first and second proteins are heterologous.

7.  A DNA construct according to Claim 4 or Claim 5 where the first and second proteins are linked by a hinge region which is a discrete region heterologous to each of said proteins.

8.  A DNA construct according to any one of the preceding Claims wherein the promoter sequence has activity which is induced by anaerobic conditions.

9.  A DNA construct according to claim 7 wherein the promoter sequence is the <u>nirB</u> promoter or a part or derivative thereof which is capable of promoting expression of a coding sequence under anaerobic conditions.

10. A DNA construct according to any one of the preceding claims wherein the second protein is an antigenic sequence which is derived from a virus, bacterium, fungus, yeast or parasite.

11. A DNA construct according to Claim 7 wherein the antigenic sequence comprises the P28 antigen of <u>Shistosoma mansoni</u> or an epitope thereof, or an antigenic sequence derived from human papilloma virus, Herpes simplex virus, foot and mouth disease virus or simian immuno-deficiency virus.

12. A DNA construct comprising a promoter sequence whose activity is induced in response to a change in the surrounding environment, said promoter sequence being operably linked to a DNA sequence encoding a first antigenic sequence and a hinge region, and at or adjacent the 3'-end thereof one or more

restriction sites for the introduction of a second antigenic sequence.

13. A DNA construct comprising a promoter sequence operably linked to a first DNA sequence encoding Tetanus toxin C fragment, or one or more epitopes thereof, and a hinge region as defined in Claim 12.

14. A DNA construct according to Claim 13 wherein the promoter is one having activity which is induced in reponse to a change in the surrounding environment.

15. A DNA construct according to any one of Claims 12 to 14 wherein the promoter sequence has activity which is induced by anaerobic conditions.

16. A DNA construct according to claim 15 wherein the promoter is the nirB promoter or a part or derivative thereof which is capable of promoting expression of a sequence under anaerobic condition.

17. A replicable expression vector, for example suitable for use in bacteria, containing a DNA construct as defined in any one of Claims 1 to 16.

18. A bacterium transformed with an expression vector as defined in claim 17.

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19.  A bacterium according to claim 18 which is attenuated.

20.  A process for the preparation of an attenuated bacterium
     according to Claim 19 which comprises transforming an
     attenuated bacterium with a DNA construct as defined in any
     one of claims 1 to 16.


21.  A fusion protein comprising first and second proteins linked
     by a hinge region, the fusion protein being expressable by a
     replicable expression vector as defined in Claim 17.


22.  A fusion protein comprising Tetanus toxin fragment C or one
     or more epitopes thereof linked to a second heterologous
     protein.


23.  A fusion protein according to Claim 22 in substantially pure
     form.


24.  A vaccine composition comprising an attenuated bacterium as
     defined in Claim 19, or a fusion protein as defined in Claim
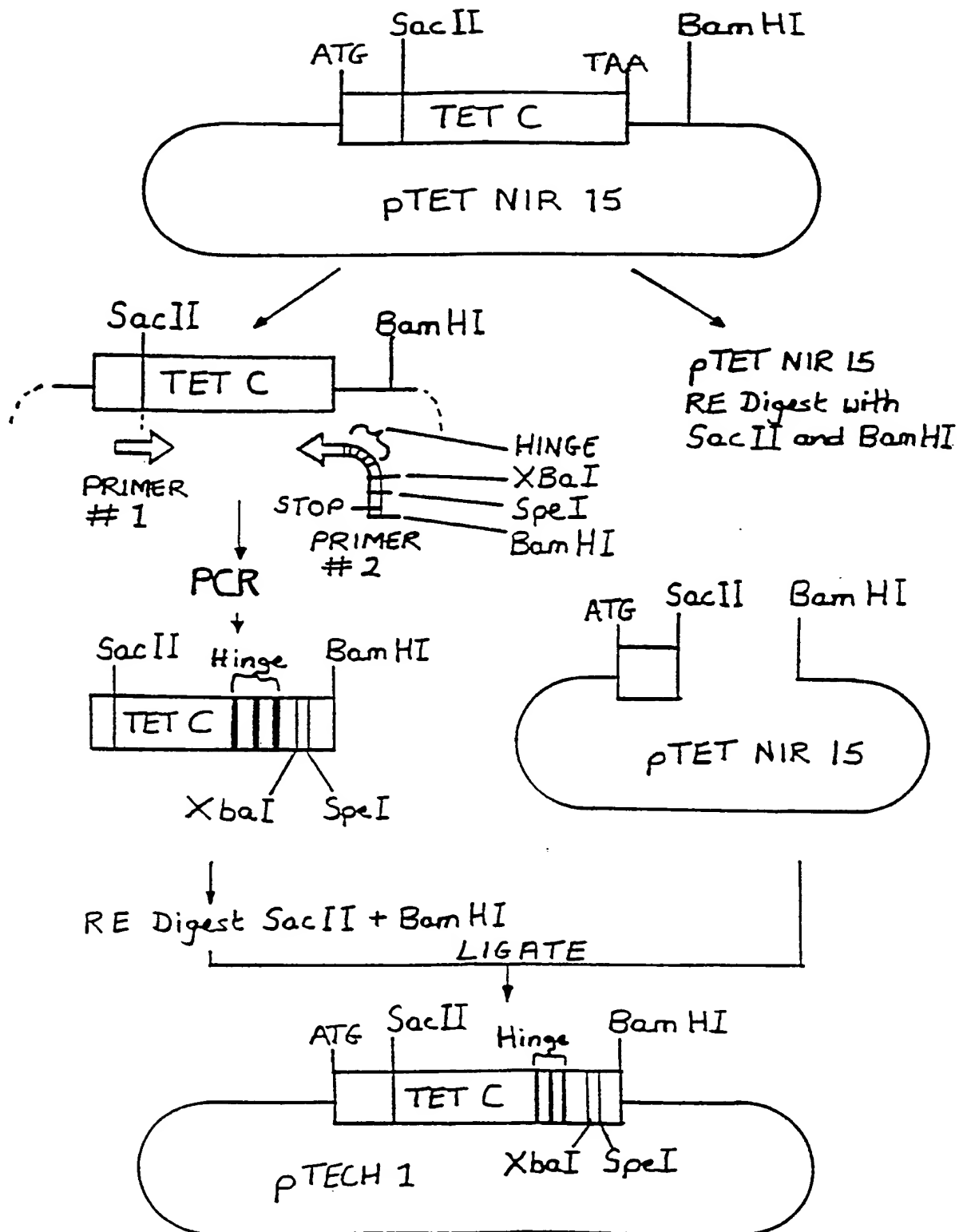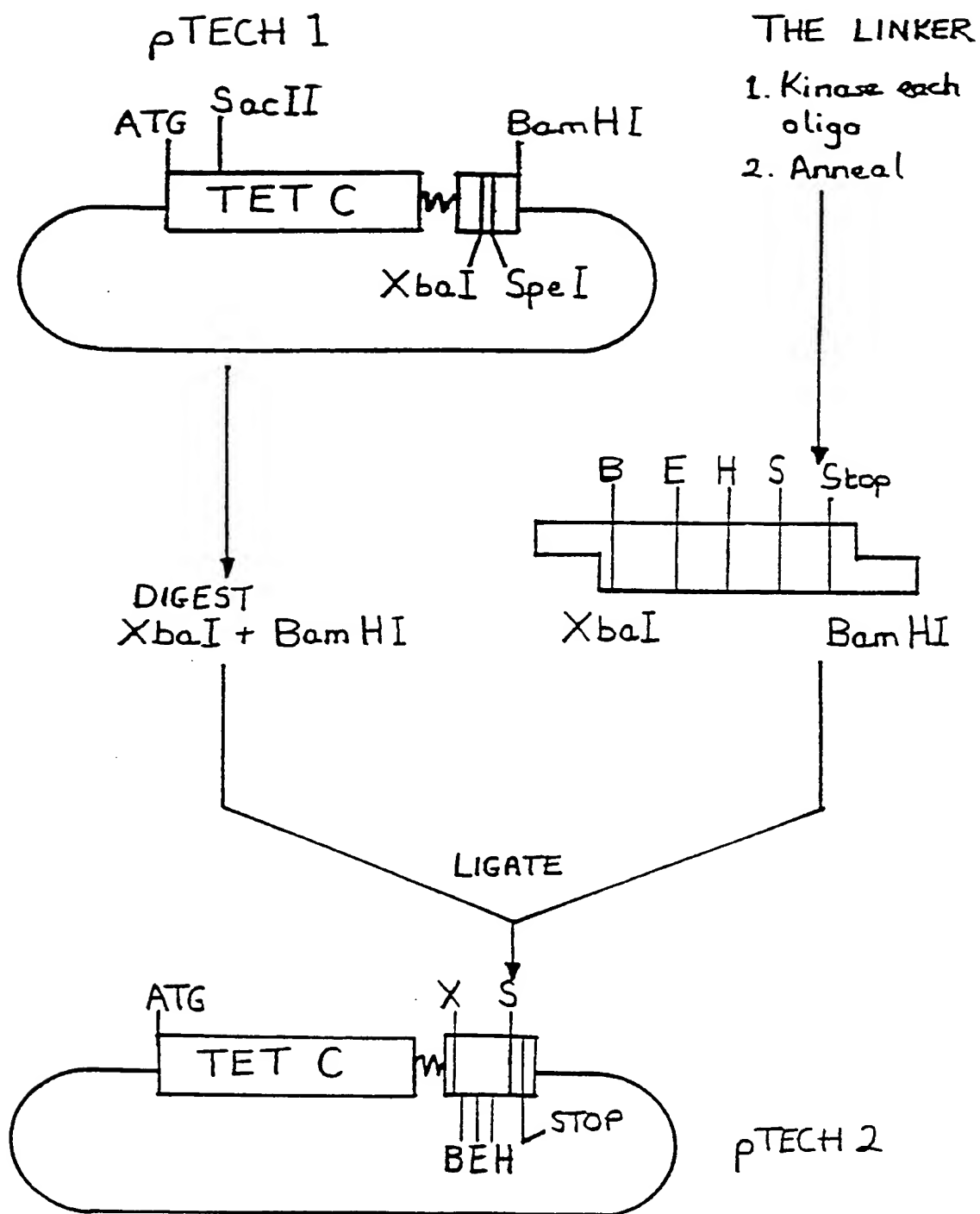     22 or Claim 23, and a pharmaceutically acceptable carrier.

FIGURE 1

FIGURE 2

FIGURE 3
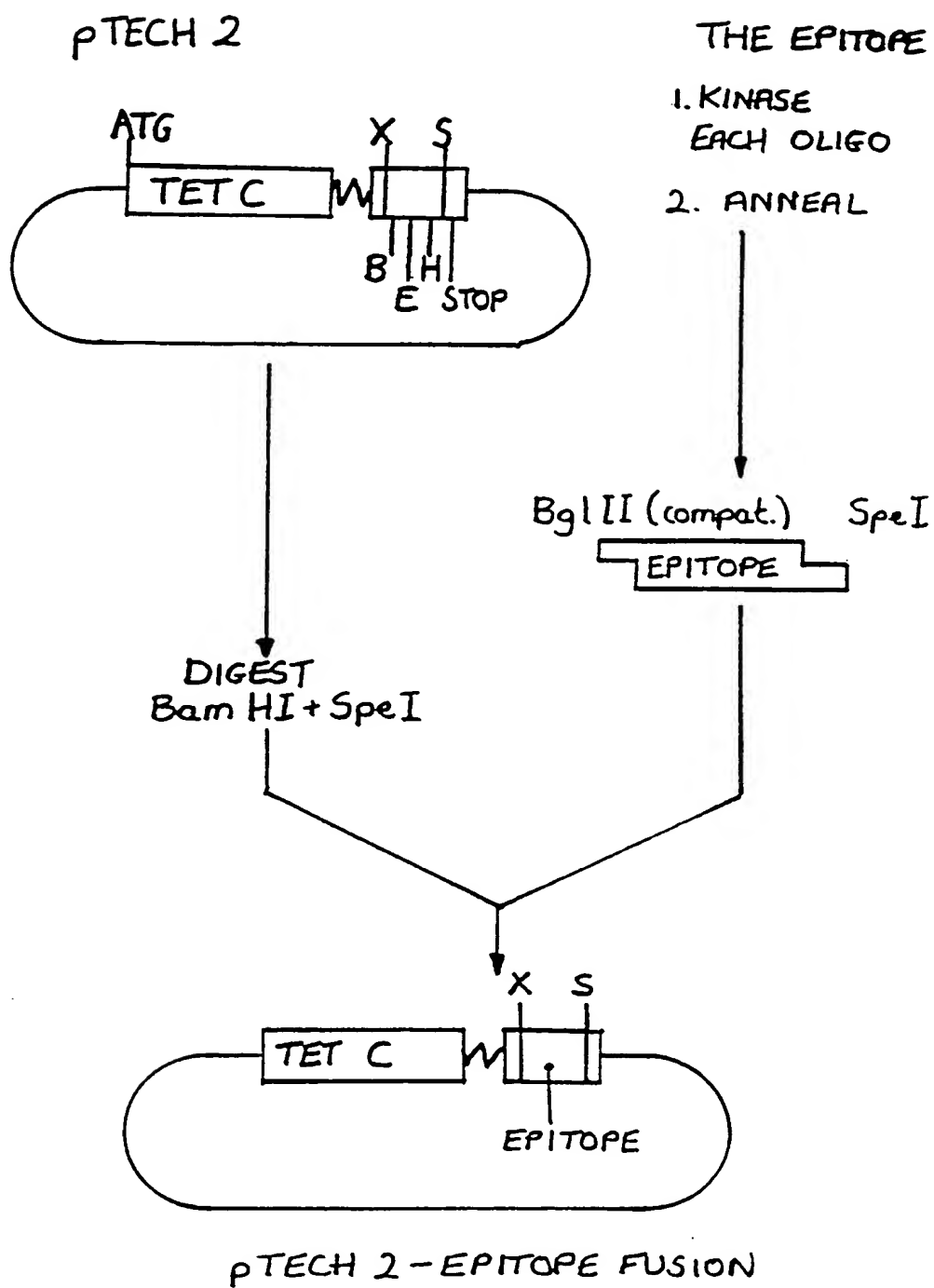
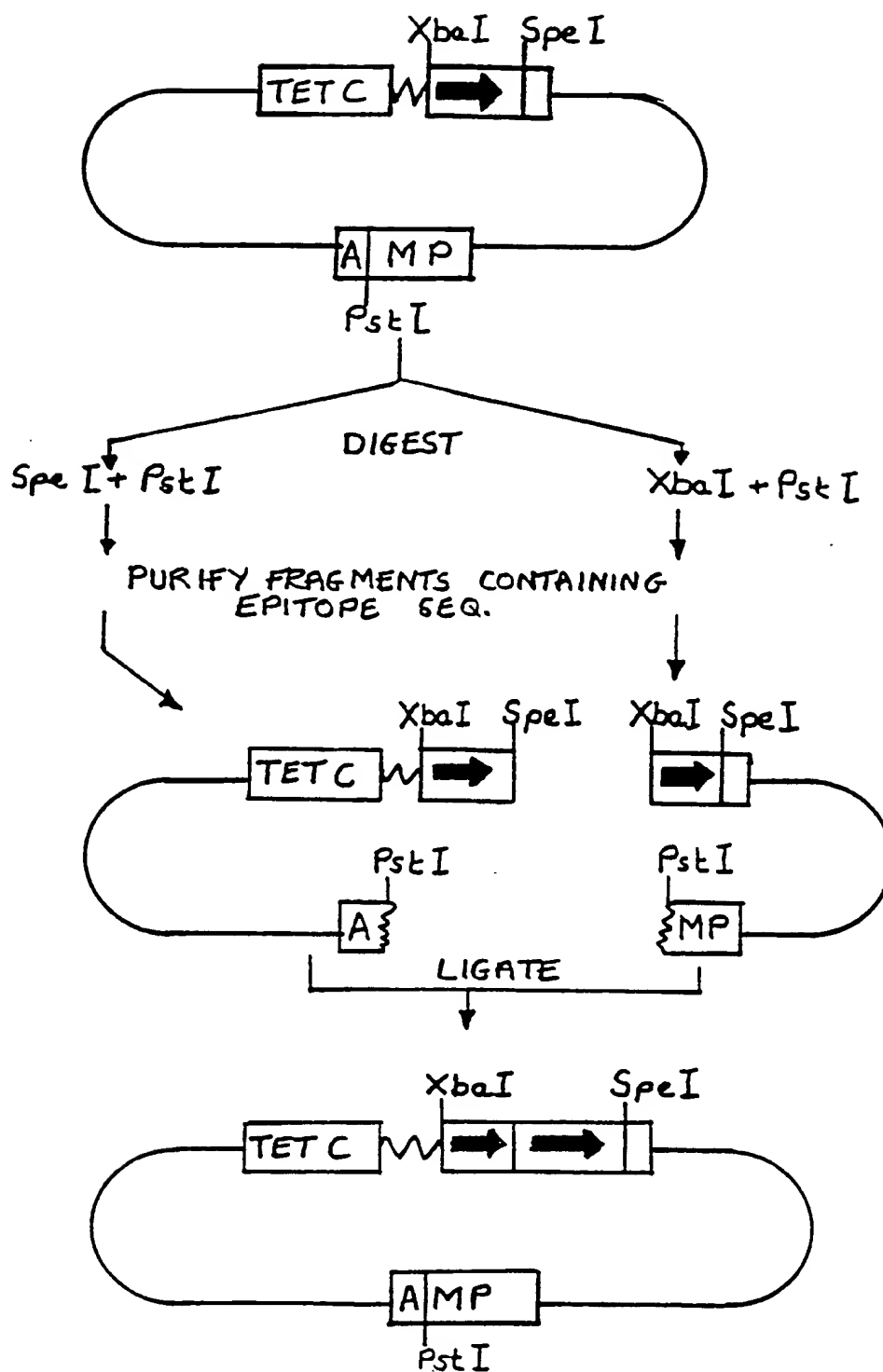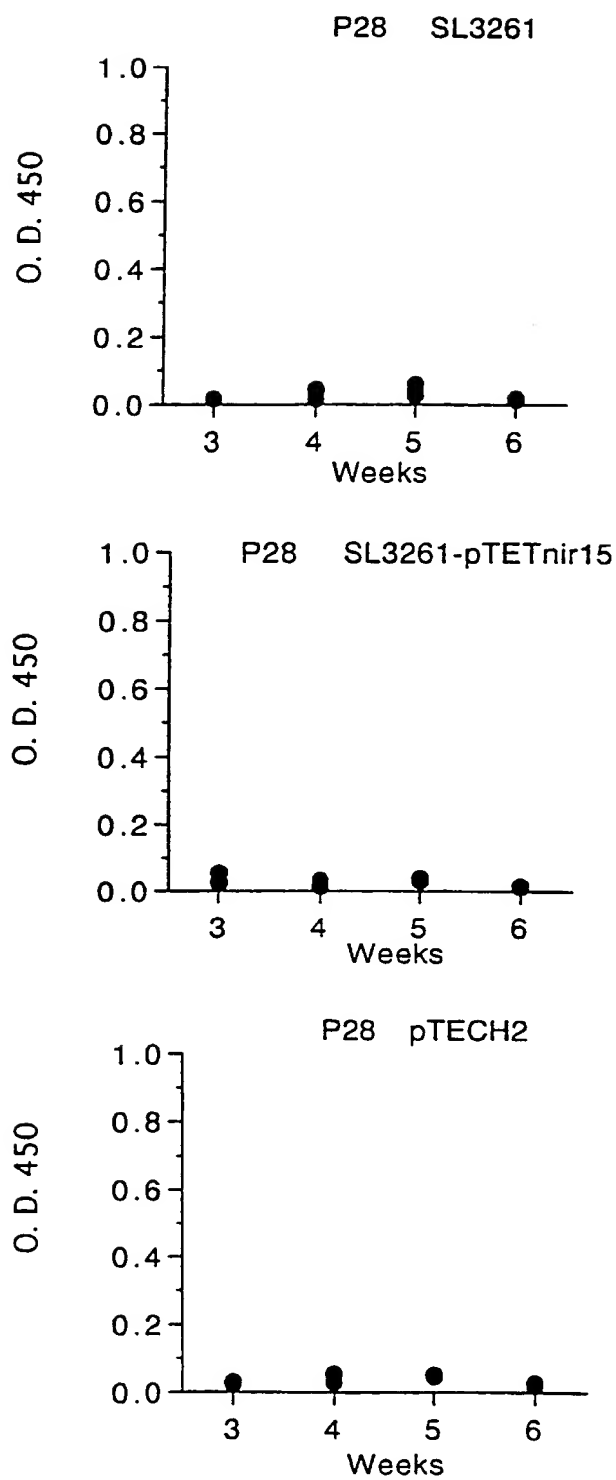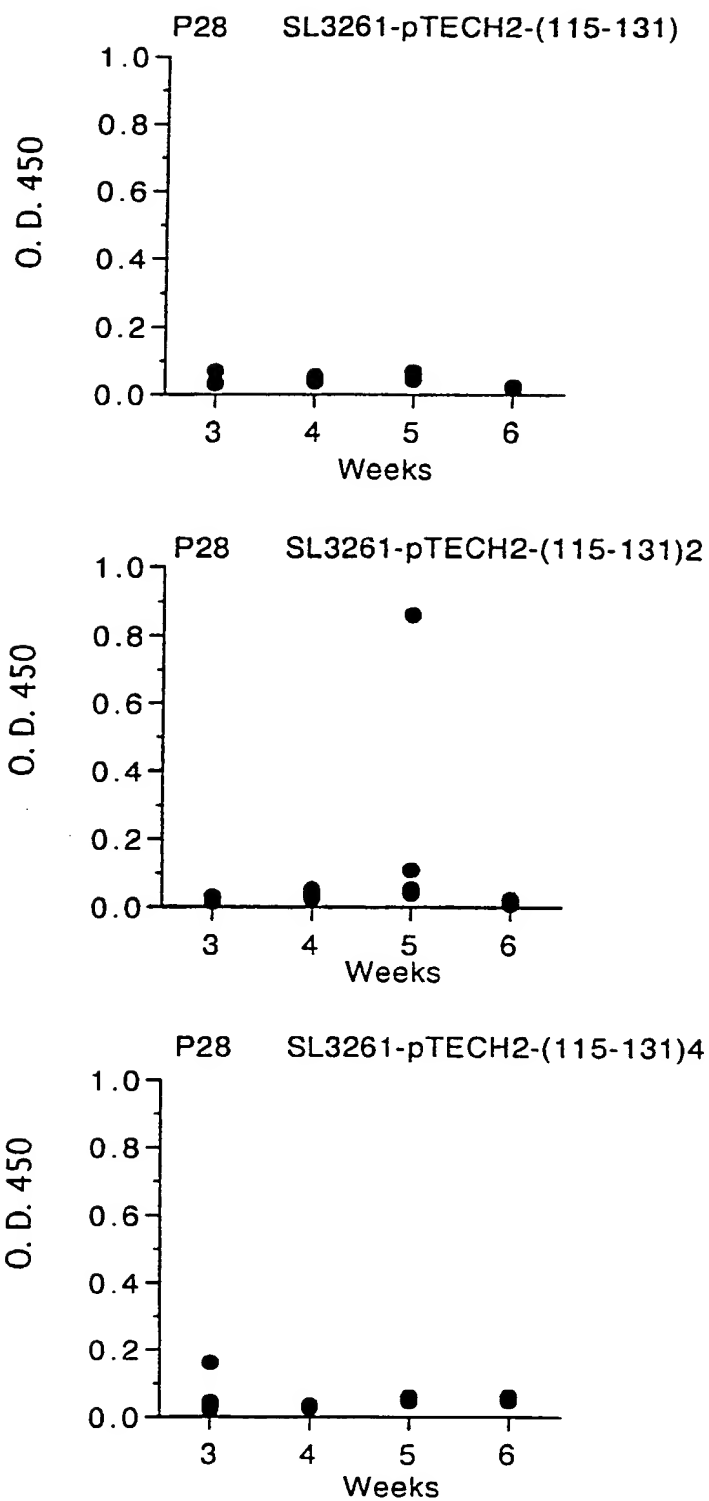FIGURE 4

Figure 5
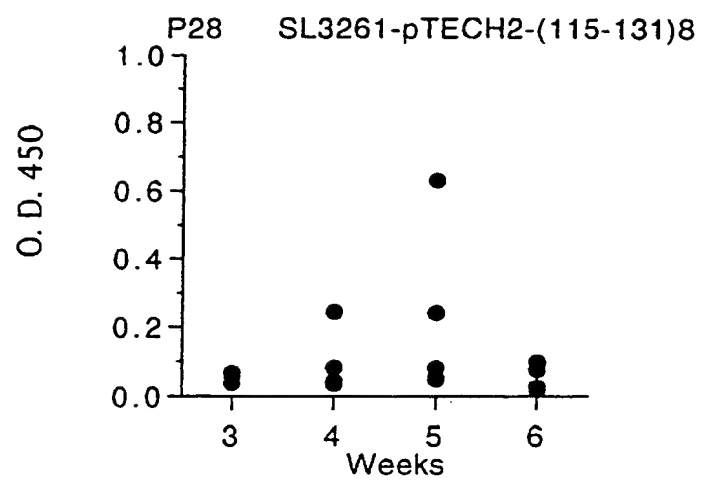
Figure 5 continued
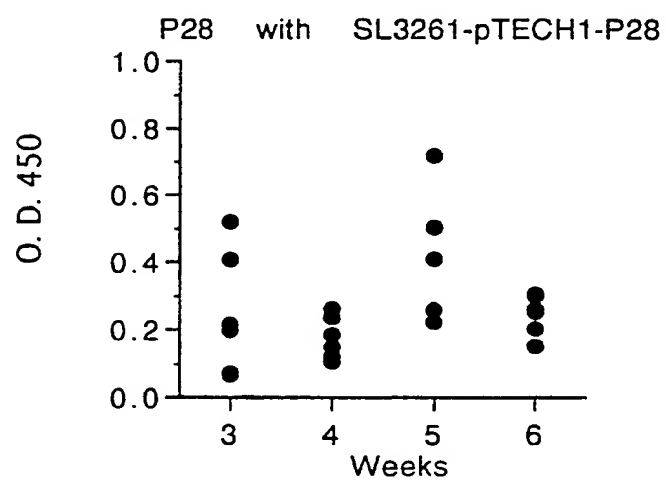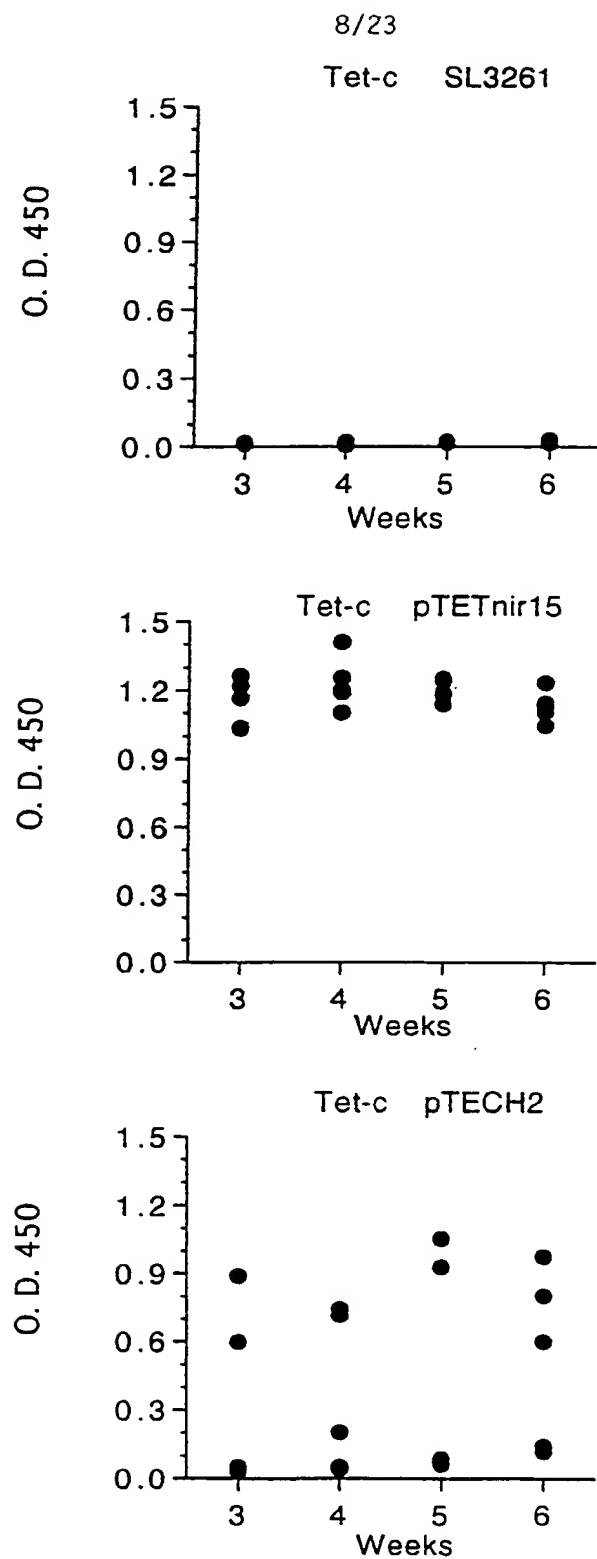
Figure 5 continued

Figure 6

Figure 6 continued

Figure 6 continued

115-131       SL3261



115-131       SL3261-pTECH2



115-131       SL3261-pTECH2-(115-131)
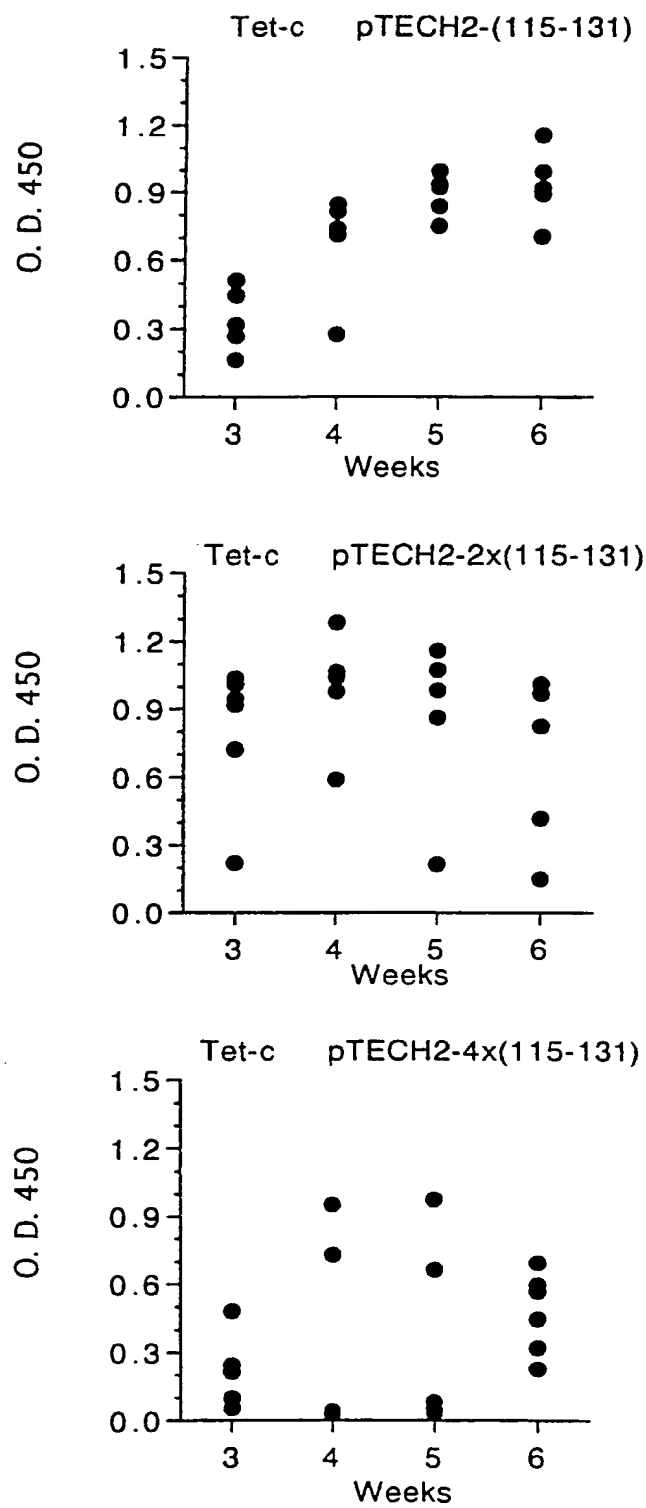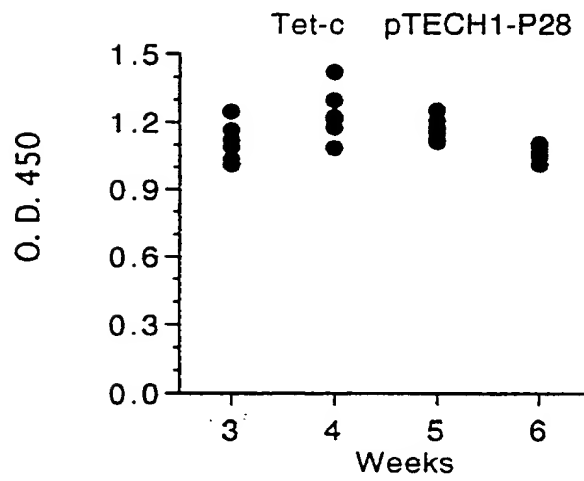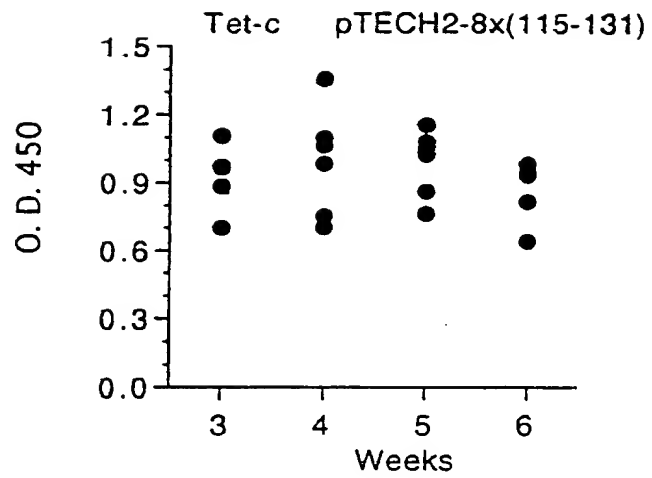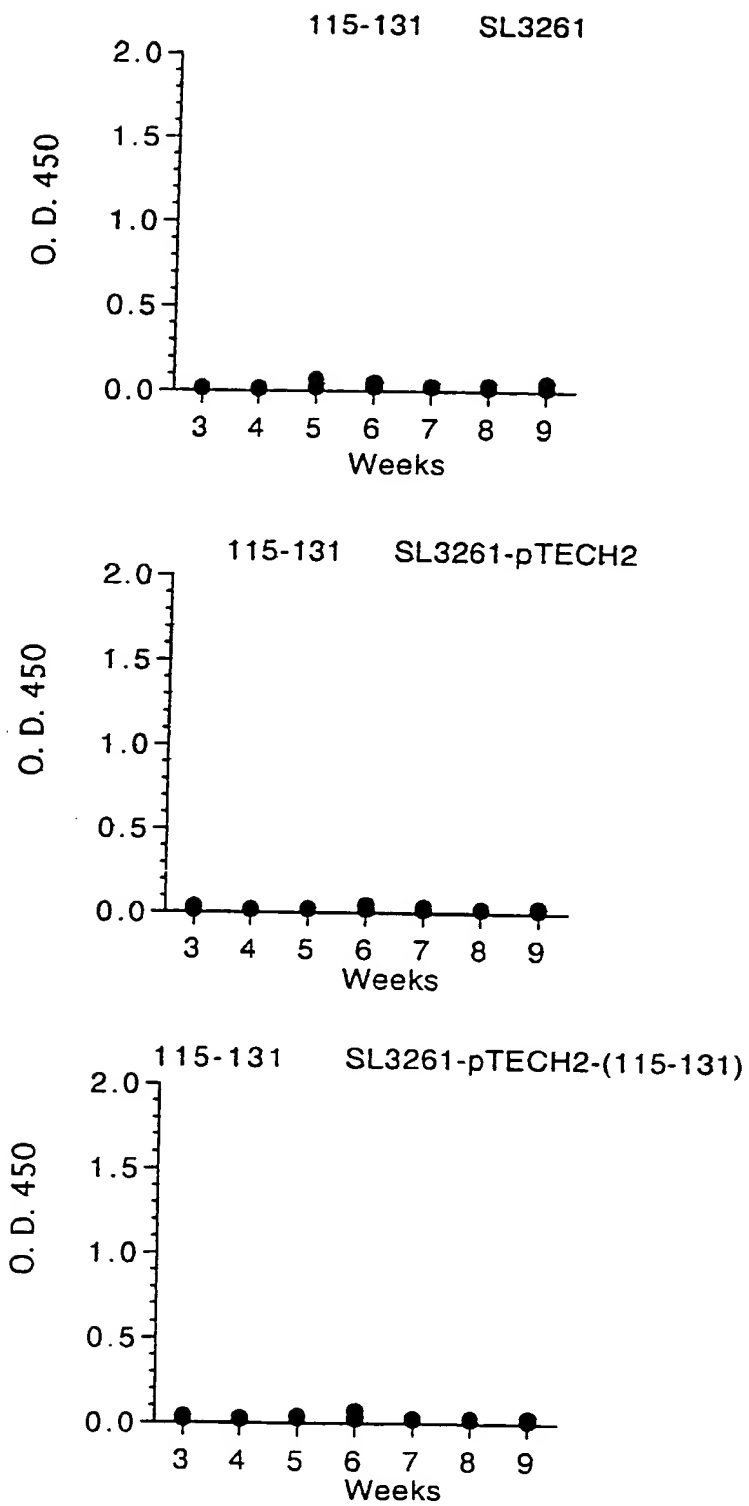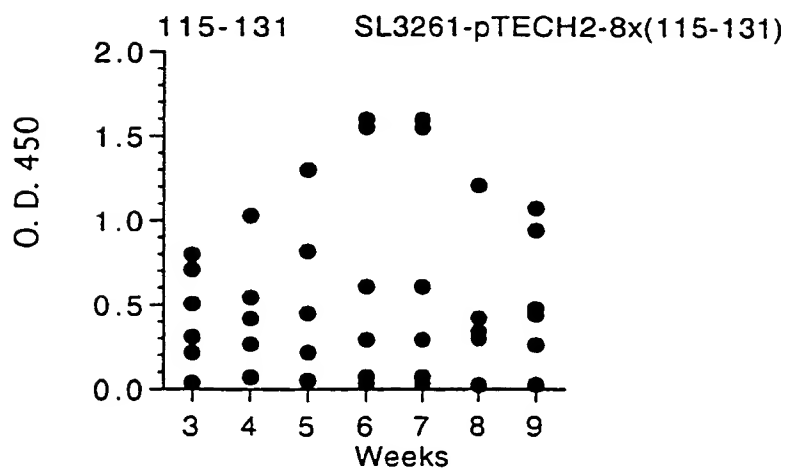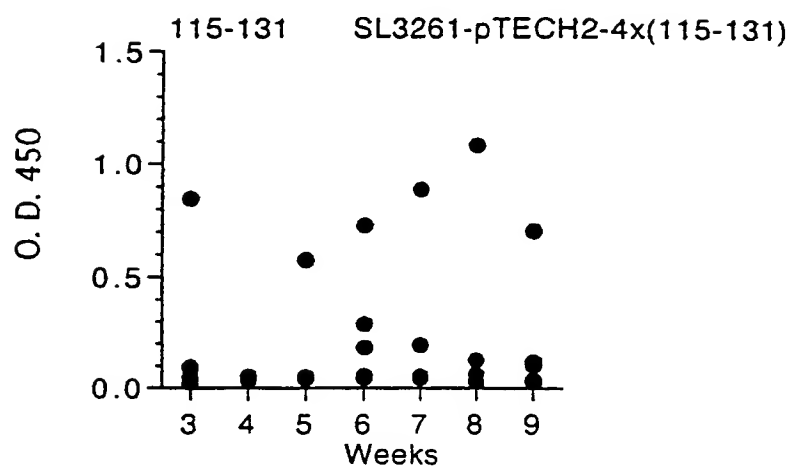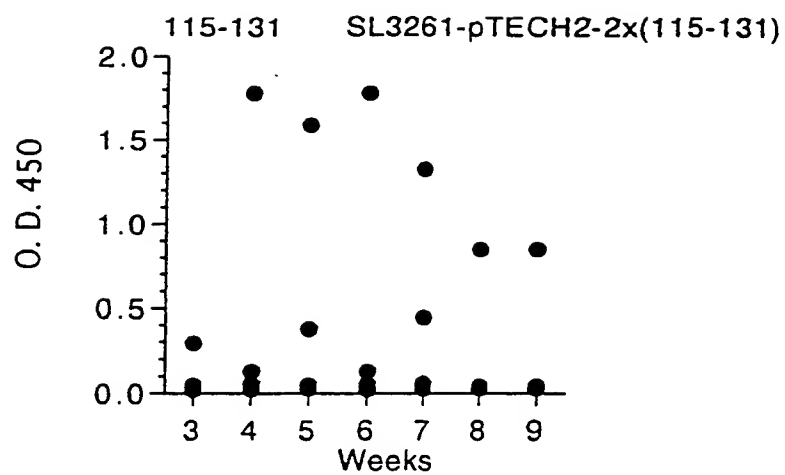


Figure 7

Figure 7 continued

Figure 8

Figure 9

## THE CONSTRUCTS

pTetNir 15

pTECH 1

pTECH1-P28 GENE

pTECH2 → pTECH2-HSVgD

pTECH2 → pTECH2-HPVE7

| **Sm P28**<br>aa 115-131 | **SIV gp120**<br>V2 LOOP | **FMDV VP1**<br>aa136-159 |
|---|---|---|
| Monomer | Monomer | Monomer |
| Dimer | Dimer | Dimer |
| Tetramer | Tetramer | Tetramer |
| Octamer | Octamer | Octamer |
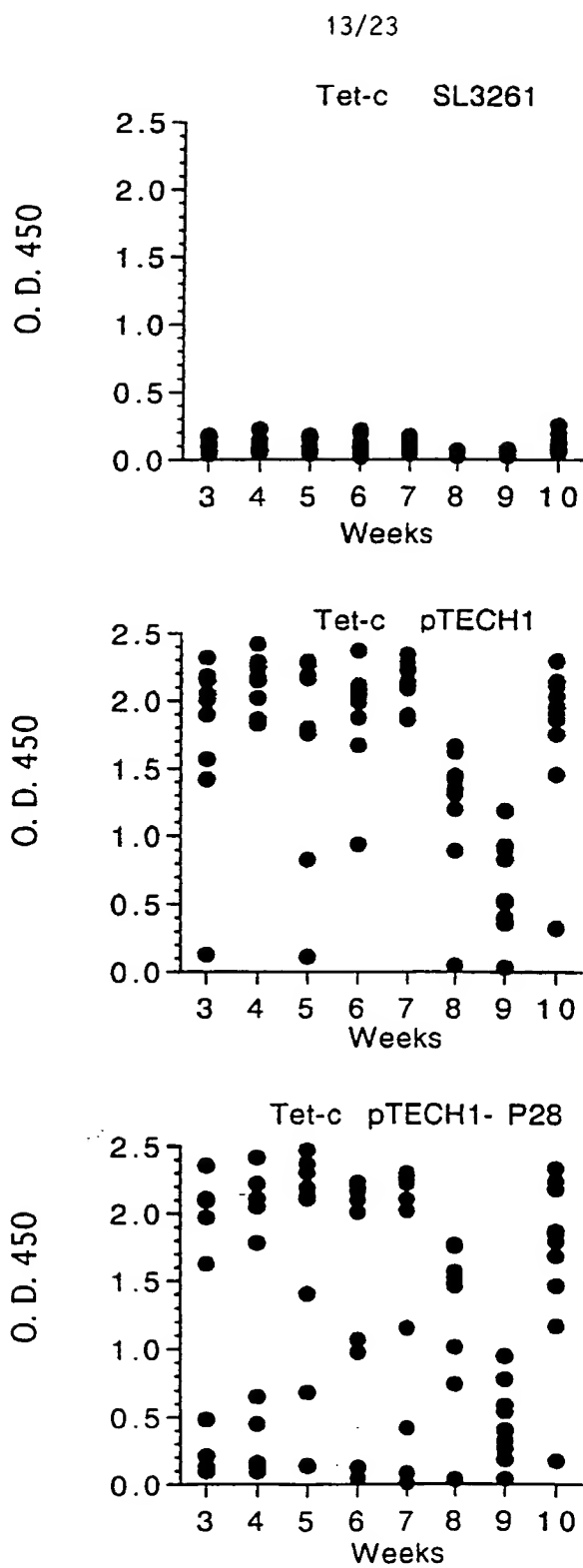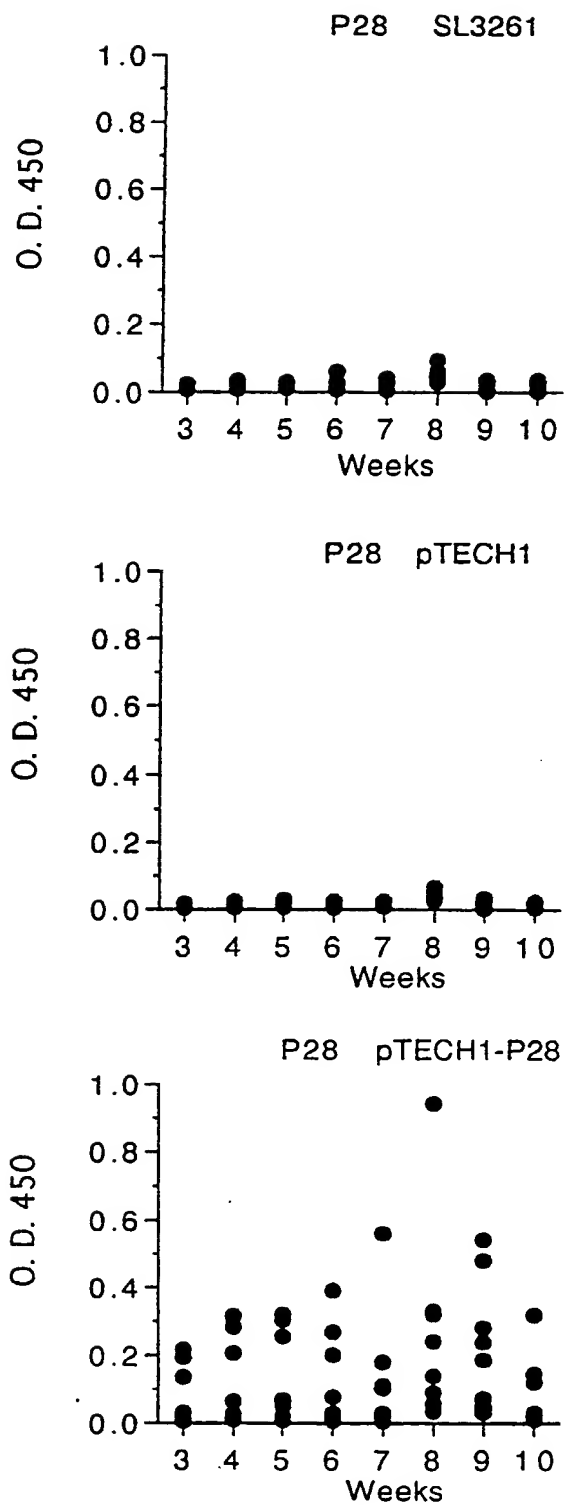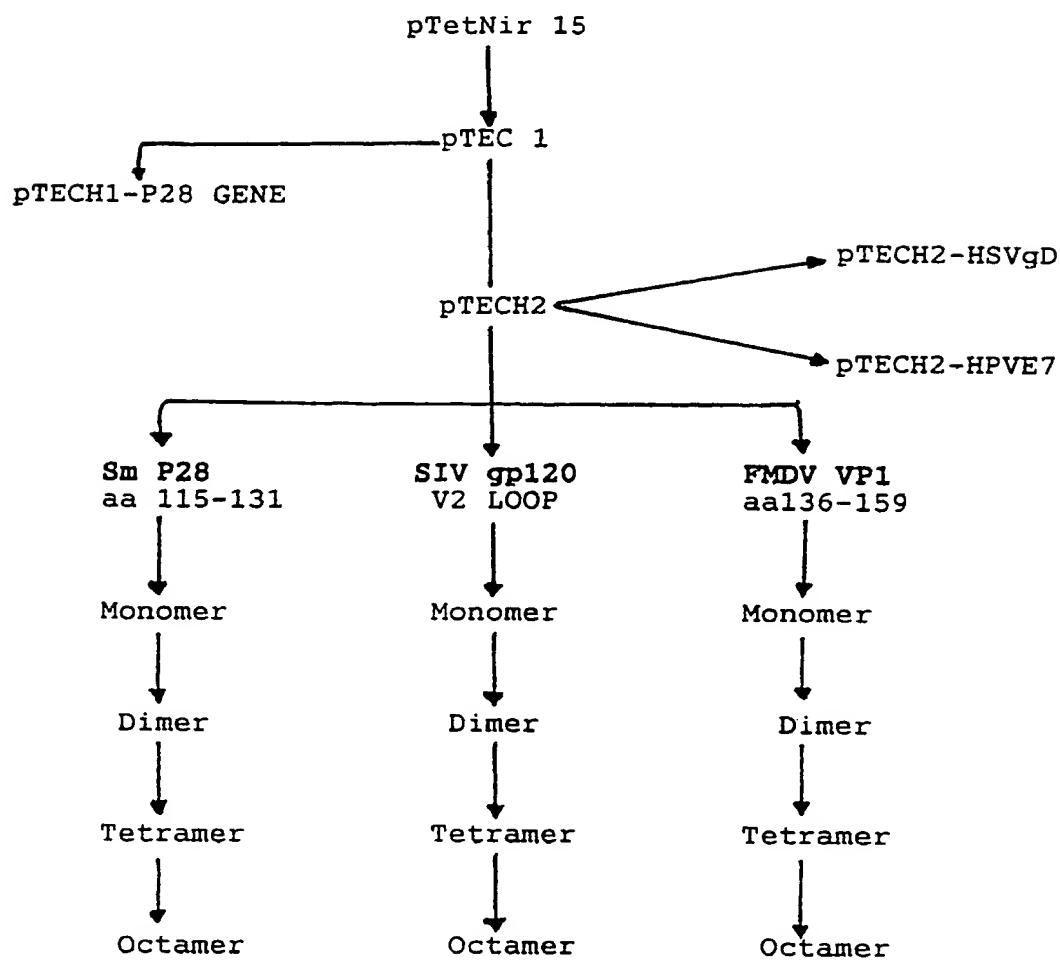
FIGURE 10

Examples of Heteromers

TET C  〰●●▲▲

TET C  〰▲▲●●

● = S. mansoni P28 epitope

▲ = SIV gp 120 V2 epitope

〰 = Hinge

FIGURE 11

FIGURE 12

DNA Sequence of the Vector pTECH1

(SEQ ID NO: 17)


1bp  -TTCAGGTAAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATCGTTAAGGTAGGCGGT - 60bp

      AGGGCCCAGATCTTAATCATCCACAGGAGACTTTCTGATGAAAAACCTTGATTGTTGGGT

      CGACAACGAAGAAGACATCGATGTTATCCTGAAAAAGTCTACCATTCTGAACTTGGACAT

      CAACAACGATATTATCTCCGACATCTCTGGTTTCAACTCCTCTGTTATCACATATCCAGA

      TGCTCAATTGGTGCCGGGCATCAACGGCAAAGCTATCCACCTGGTTAACAACGAATCTTC

      TGAAGTTATCGTGCACAAGGCCATGGACATCGAATACAACGACATGTTCAACAACTTCAC

      CGTTAGCTTCTGGCTGCGCGTTCCGAAAGTTTCTGCTTCCCACCTGGAACAGTACGGCAC

      TAACGAGTACTCCATCATCAGCTCTATGAAGAAACACTCCCTGTCCATCGGCTCTGGTTG

      GTCTGTTTCCCTGAAGGGTAACAACCTGATCTGGACTCTGAAAGACTCCGCGGGCGAAGT

      TCGTCAGATCACTTTCCGCGACCTGCCGGACAAGTTCAACGCGTACCTGGCTAACAAATG

      GGTTTTCATCACTATCACTAACGATCGTCTGTCTTCTGCTAACCTGTACATCAACGGCGT

      TCTGATGGGCTCCGCTGAAATCACTGGTCTGGGCGCTATCCGTGAGGACAACAACATCAC

      TCTTAAGCTGGACCGTTGCAACAACAACAACCAGTACGTATCCATCGACAAGTTCCGTAT

      CTTCTGCAAAGCACTGAACCCGAAAGAGATCGAAAAACTGTATACCAGCTACCTGTCTAT

      CACCTTCCTGCGTGACTTCTGGGGTAACCCGCTGCGTTACGACACCGAATATTACCTGAT

      CCCGGTAGCTTCTAGCTCTAAAGACGTTCAGCTGAAAAACATCACTGACTACATGTACCT

      GACCAACGCGCCGTCCTACACTAACGGTAAACTGAACATCTACTACCGACGTCTGTACAA

      CGGCCTGAAATTCATCATCAAACGCTACACTCCGAACAACGAAATCGATTCTTTCGTTAA

      ATCTGGTGACTTCATCAAACTGTACGTTTCTTACAACAACAACGAACACATCGTTGGTTA

      CCCGAAAGACGGTAACGCTTTCAACAACCTGGACAGAATTCTGCGTGTTGGTTACAACGC

      TCCGGGTATCCCGCTGTACAAAAAAATGGAAGCTGTTAAACTGCGTGACCTGAAAACCTA

      CTCTGTTCAGCTGAAACTGTACGACGACAAAAACGCTTCTCTGGGTCTGGTTGGTACCCA

      CAACGGTCAGATCGGTAACGACCCGAACCGTGACATCCTGATCGCTTCTAACTGGTACTT

      CAACCACCTGAAAGACAAAATCCTGGGTTGCGACTGGTACTTCGTTCCGACCGATGAAGG

      TTGGACCAACGACGGGCCGGGGCCCTCTAGAATCACTAGTTAAGGATCCGCTAGCCCGCC

pTECH1 DNA Sequence continued

TAATGAGCGGGCTTTTTTTTTCTCGGGCAGCGTTGGGTCCTGGCCACGGGTGCGCATGATC

GTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCGGGGTTGCCTTACTGGTTAGCAGAAT

GAATCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACGTCTGCGACCTGA

GCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAGTCTGGAAACGCGGAAGTCA

GCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGC

GGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGG

AAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCT

GGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCA

GAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCT

CGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTC

GGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGT

TCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATC

CGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGC

CACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTG

GTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCC

AGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAG

CGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGA

TCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGAT

TTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAG

TTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAAT

CAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCC

CGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGAT

ACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAG

GGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTG

pTECH1 DNA Sequence  continued


CCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGC

TGCAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCA

ACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGG

TCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGC

ACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTA

CTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTC

AACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACG

TTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACC

CACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGC

AAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAAT

ACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG

CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCC

CCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAA

TAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAA  - 3754bp

## FIGURE 13

### DNA Sequence of the Vector pTECH2

(SEQ ID NO: 18)

1bp -   TTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATCGTTAAGGTAGGCGGT - 60bp

AGGGCCCAGATCTTAATCATCCACAGGAGACTTTCTGATGAAAAACCTTGATTGTTGGGT

CGACAACGAAGAAGACATCGATGTTATCCTGAAAAAGTCTACCATTCTGAACTTGGACAT

CAACAACGATATTATCTCCGACATCTCTGGTTTCAACTCCTCTGTTATCACATATCCAGA

TGCTCAATTGGTGCCGGGCATCAACGGCAAAGCTATCCACCTGGTTAACAACGAATCTTC

TGAAGTTATCGTGCACAAGGCCATGGACATCGAATACAACGACATGTTCAACAACTTCAC

CGTTAGCTTCTGGCTGCGCGTTCCGAAAGTTTCTGCTTCCCACCTGGAACAGTACGGCAC

TAACGAGTACTCCATCATCAGCTCTATGAAGAAACACTCCCTGTCCATCGGCTCTGGTTG

GTCTGTTTCCCTGAAGGGTAACAACCTGATCTGGACTCTGAAAGACTCCGCGGGCGAAGT

TCGTCAGATCACTTTCCGCGACCTGCCGGACAAGTTCAACGCGTACCTGGCTAACAAATG

GGTTTTCATCACTATCACTAACGATCGTCTGTCTTCTGCTAACCTGTACATCAACGGCGT

TCTGATGGGCTCCGCTGAAATCACTGGTCTGGGCGCTATCCGTGAGGACAACAACATCAC

TCTTAAGCTGGACCGTTGCAACAACAACAACCAGTACGTATCCATCGACAAGTTCCGTAT

CTTCTGCAAAGCACTGAACCCGAAAGAGATCGAAAAACTGTATACCAGCTACCTGTCTAT

CACCTTCCTGCGTGACTTCTGGGGTAACCCGCTGCGTTACGACACCGAATATTACCTGAT

CCCGGTAGCTTCTAGCTCTAAAGACGTTCAGCTGAAAAACATCACTGACTACATGTACCT

GACCAACGCGCCGTCCTACACTAACGGTAAACTGAACATCTACTACCGACGTCTGTACAA

CGGCCTGAAATTCATCATCAAACGCTACACTCCGAACAACGAAATCGATTCTTTCGTTAA

ATCTGGTGACTTCATCAAACTGTACGTTTCTTACAACAACAACGAACACATCGTTGGTTA

CCCGAAAGACGGTAACGCTTTCAACAACCTGGACAGAATTCTGCGTGTTGGTTACAACGC

TCCGGGTATCCCGCTGTACAAAAAAATGGAAGCTGTTAAACTGCGTGACCTGAAAACCTA

CTCTGTTCAGCTGAAACTGTACGACGACAAAAACGCTTCTCTGGGTCTGGTTGGTACCCA

CAACGGTCAGATCGGTAACGACCCGAACCGTGACATCCTGATCGCTTCTAACTGGTACTT

CAACCACCTGAAAGACAAAATCCTGGGTTGCGACTGGTACTTCGTTCCGACCGATGAAGG

TTGGACCAACGACGGCCGGGCCCTCTAGAGGATCCGATATCAAGCTTACTAGTTAATG

ATCCGCTAGCCCGCCTAATGAGCGGGCTTTTTTTTTCTCGGGCAGCGTTGGGTCCTGGCCA

CGGGTGCGCATGATCGTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCGGGGTTGCCTT

pTECH2 DNA Sequence continued

ACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAA

ACGTCTGCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAGTCTG

GAAACGCGGAAGTCAGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGT

TCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC

AGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAA

AAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAA

TCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC

CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTC

CGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAG

TTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGA

CCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATC

GCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTAC

AGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTG

CGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACA

AACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAA

AGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA

CTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTT

AAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAG

TTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCAT

AGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCC

CAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAA

CCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCA

GTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAA

CGTTGTTGCCATTGCTGCAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATT

CAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGC

GGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACT

CATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC

pTECH2 DNA Sequence continued

TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTG

CTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCT

CATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATC

CAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAG

CGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGAC

ACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGG

TTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGT

TCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGAC

ATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAA

|  | Xbal | BamHi | EcoRV | Hind111 | SpeI |  | Stop | BamHi |
|---|---|---|---|---|---|---|---|---|

```
---HINGE----   TCTAGA GGATCC GATATC AAGCTT ACTAGT TAA TGATC
               AGATCT CCTAGG CTATAG TTCGAA TGATCA ATT ACTAG
                            (SEQ ID NO: 19)

---GPGP ----      S  R  G  S  D  I  K  L  T  S  *

                       (SEQ ID NO: 20)
```

FIGURE 14

SUBSTITUTE SHEET

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE


INTERNATIONAL FORM

| | |
|---|---|
| TO ⌐ ¬<br><br>Medeva Holdings BV<br>Churchill-Laan 223<br>Amsterdam 1078-ED<br><br>⌐ NAME AND ADDRESS ¬<br>⌐ OF DEPOSITOR ¬ | RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT<br>issued pursuant to Rule 7.1 by the<br>INTERNATIONAL DEPOSITARY AUTHORITY<br>identified at the bottom of this page<br><br>Attention:  Dr S N Chatfield<br>Vaccine Research Unit<br>Imperial College of Science & Technology<br>Department of Biochemistry<br>London SW7 2AY |

---

**I.   IDENTIFICATION OF THE MICROORGANISM**

Identification reference given by the
DEPOSITOR:

  Salmonella typhimurium BRD991

Accession number given by the
INTERNATIONAL DEPOSITARY AUTHORITY:

    NCTC 12831

---

**II.   SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

The microorganism identified under I above was accompanied by:

  [   ]   a scientific description

  [ X ]   a proposed taxonomic designation

(Mark with a cross where applicable)

---

**III.   RECEIPT AND ACCEPTANCE**

This International Depositary Authority accepts the microorganism identified under I above,
which was received by it on  15 July 1993 (date of the original deposit)[1]

---

**IV.   RECEIPT OF REQUEST FOR CONVERSION       N/A**

The microorganism identified under I above was received by this International
Depositary Authority on                         (date of the original deposit) and
a request to convert the original deposit to a deposit under the Budapest Treaty
was received by it on                         (date of receipt of request for conversion)

---

**V.   INTERNATIONAL DEPOSITARY AUTHORITY**

| | |
|---|---|
| Name:  National Collection of Type Cultures<br>       Central Public Health Laboratory | Signature(s) of person(s) having the power<br>to represent the International Depositary<br>Authority or of authorized official(s) |
| Address:   61 Colindale Avenue<br>        London NW9 5HT | Date:   7 September 1993  _L. Rull_<br>                           R Hill<br>                           Curator |

[1]   Where Rule 6.4(d) applies, such date is the date on which the status of international depositary
    authority was acquired.

Form BP/4   (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

| TO<br>Medeva Holdings BV<br>Churchill-Laan 223<br>Amsterdam 1078-ED<br><br>NAME AND ADDRESS<br>OF DEPOSITOR | RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT<br>issued pursuant to Rule 7.1 by the<br>INTERNATIONAL DEPOSITARY AUTHORITY<br>identified at the bottom of this page<br><br>Attention:  Dr S N Charfield<br>Vaccine Research Unit<br>Imperial College of Science & Technology<br>Department of Biochemistry<br>London SW7 2AY |
|---|---|

---

**I.  IDENTIFICATION OF THE MICROORGANISM**

Identification reference given by the DEPOSITOR:

Salmonella typhimurium BRD992

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:

NCTC 12832

---

**II.  SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

The microorganism identified under I above was accompanied by:

☐  a scientific description

☒  a proposed taxonomic designation

(Mark with a cross where applicable)

---

**III.  RECEIPT AND ACCEPTANCE**

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on  15 July 1993 (date of the original deposit)[1]

---

**IV.  RECEIPT OF REQUEST FOR CONVERSION    N/A**

The microorganism identified under I above was received by this International Depositary Authority on                        (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on                        (date of receipt of request for conversion)

---

**V.  INTERNATIONAL DEPOSITARY AUTHORITY**

| Name:  National Collection of Type Cultures<br>Central Public Health Laboratory | Signature(s) of person(s) having the power<br>to represent the International Depositary<br>Authority or of authorized official(s): |
|---|---|
| Address:  61 Colindale Avenue<br>London NW9 5HT | Date:  7 September 1993   L.R. Hill<br>Curator |

---

[1]    Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4   (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

| | |
|---|---|
| TO<br><br>Medeva Holdings BV<br>Churchill-Laan 223<br>Amsterdam 1078-ED<br><br>NAME AND ADDRESS<br>OF DEPOSITOR | RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT<br>issued pursuant to Rule 7.1 by the<br>INTERNATIONAL DEPOSITARY AUTHORITY<br>identified at the bottom of this page<br><br>Attention: Dr S N Chatfield<br>Vaccine Research Unit<br>Imperial College of Science & Technology<br>Department of Biochemistry<br>London SW7 2AY |

---

**I. IDENTIFICATION OF THE MICROORGANISM**

| Identification reference given by the DEPOSITOR: | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: |
|---|---|
| Salmonella typhimurium BRD993 | NCTC 12833 |

---

**II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

The microorganism identified under I above was accompanied by:

|   | a scientific description

| X | a proposed taxonomic designation

(Mark with a cross where applicable)

---

**III. RECEIPT AND ACCEPTANCE**

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on   15 July 1993(date of the original deposit)[1]

---

**IV. RECEIPT OF REQUEST FOR CONVERSION   N/A**

The microorganism identified under I above was received by this International Depositary Authority on                    (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on                     (date of receipt of request for conversion)

---

**V. INTERNATIONAL DEPOSITARY AUTHORITY**

| | |
|---|---|
| Name: National Collection of Type Cultures<br>Central Public Health Laboratory<br><br>Address: 61 Colindale Avenue<br>London NW9 5HT | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):<br><br>Date: 7 September 1993   L R Hill<br>Curator |

[1]   Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE


INTERNATIONAL FORM

```
┌ TO                            ┐
  Medeva Holdings BV
  Churchill-Laan 223
  Amsterdam 1078-ED

  ┌   NAME AND ADDRESS          ┐
  └      OF DEPOSITOR           ┘
```

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

Attention:   Dr S N Chatfield
             Vaccine Research Unit
             Imperial College of Science & Technology
             Department of Biochemistry
             London SW7 2AY

---

**I.   IDENTIFICATION OF THE MICROORGANISM**

| Identification reference given by the DEPOSITOR: | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: |
|---|---|
| Salmonella typhimurium BRD994 | NCTC 12834 |

---

**II.   SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

The microorganism identified under I above was accompanied by:

☐    a scientific description

☒    a proposed taxonomic designation

(Mark with a cross where applicable)

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**III.   RECEIPT AND ACCEPTANCE**

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 15 July 1993 (date of the original deposit)[1]

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**IV.   RECEIPT OF REQUEST FOR CONVERSION    N/A**

The microorganism identified under I above was received by this International Depositary Authority on                         (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on                         (date of receipt of request for conversion)

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**V.   INTERNATIONAL DEPOSITARY AUTHORITY**

| | |
|---|---|
| Name:   National Collection of type Cultures Central Public Health Laboratory | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): |
| Address:   61 Colindale Avenue London NW9 5HT | Date:   7 September 1993   R Hill  Curator |

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[1]    Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4   (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE


INTERNATIONAL FORM

TO
Medeva Holdings BV
Churchill-Laan 223
Amsterdam 1078-ED

NAME AND ADDRESS
OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

Attention: Dr S N Chatfield
Vaccine Research Unit
Imperial College of Science & Technology
Department of Biochemistry
London SW7 2AY

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**I. IDENTIFICATION OF THE MICROORGANISM**

| Identification reference given by the DEPOSITOR: | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: |
|---|---|
| Escherichia coli BRD970 | NCTC 12837 |

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**II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

The microorganism identified under I above was accompanied by:

[ ]   a scientific description

[X]   a proposed taxonomic designation

(Mark with a cross where applicable)

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**III. RECEIPT AND ACCEPTANCE**

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on  23 July 1993 (date of the original deposit)[1]

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**IV. RECEIPT OF REQUEST FOR CONVERSION     N/A**

The microorganism identified under I above was received by this International Depositary Authority on                          (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on                          (date of receipt of request for conversion)

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**V. INTERNATIONAL DEPOSITARY AUTHORITY**

| Name: National Collection of Type Cultures Central Public Health Laboratory | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): |
|---|---|
| Address: 61 Colindale Avenue London NW9 5HT | Date: 7 September 1993    L R Hill  Curator |

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[1]   Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4   (sole page)

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/62;    C12N15/31;    C12N15/54;    C12N1/21
    C07K13/00;    A61K39/08;    //(C12N1/21,C12R1:42)

**II. FIELDS SEARCHED**

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | C12N ;    C07K ;    A61K |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**[9]

| Category[o] | Citation of Document,[11] with indication, where appropriate, of the relevant passages[12] | Relevant to Claim No.[13] |
|---|---|---|
| Y | NATURE<br>vol. 330, 12 November 1987, MACMILLAN JOURNALS LTD., LONDON, UK;<br>pages 168 - 170<br>M.J. FRANCIS ET AL. 'Non-responsiveness to a foot-and-mouth disease virus peptide overcome by addition of foreign helper T-cell determinants'<br>see page 168, left column, line 1 - page 170, right column, line 18<br>--- | 1-24 |
| Y | WO,A,8 906 974 (PRAXIS BIOLOGICS, INC.)<br>10 August 1989<br>see page 25, line 12 - page 26, line 9<br>see page 26, line 25 - page 27, line 14<br>see page 76, line 1 - page 81, line 4;<br>claims 1-107; tables 12-15<br>--- | 1-24 |

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 14 OCTOBER 1993 | 22. 10. 93 |

| International Searching Authority | Signature of Authorized Officer |
|---|---|
| EUROPEAN PATENT OFFICE | HORNIG H. |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT | (CONTINUED FROM THE SECOND SHEET) | |
|---|---|---|
| Category° | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
| Y | EP,A,0 432 965 (SMITHKLINE BEECHAM) 19 June 1991 see page 16, line 24 - line 29 --- | 1-24 |
| Y | NUCLEIC ACIDS REASEARCH vol. 19, no. 11, 11 June 1991, IRL,OXFORD UNIVERSITY PRESS, UK; pages 2889 - 2892 M.D. OXER ET AL. 'High level heterologous expression in E. coli using the anaerobically-activated nirB promoter' cited in the application see page 2890, left column, paragraph 4 - page 2892, right column, line 19 --- | 1-24 |
| Y | WO,A,9 109 621 (INSTITUT PASTEUR) 11 July 1991 see page 2, line 12 - line 15 --- | 1-24 |
| Y | J. IMMUNOLOGY vol. 141, no. 5, 1 September 1988, AM. SOC. IMMUNOLOGISTS,US; pages 1687 - 1694 C. AURIAULT ET AL. 'Analysis of T and B cell epitopes of the Schistosoma mansoni P28 antigen in the rat model by using synthetic peptides' see page 1688, left column, line 18 - line 29 --- | 1-24 |
| P,Y | WO,A,9 215 689 (THE WELLCOME FOUNDATION LIMITED) 17 September 1992 see page 8, line 2 - page 9, line 26; claims 1-12 see page 11, line 20 - line 35 ----- | 1-24 |